PCT

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 : G01N 27/26, 27/447	**A1**	**(11) International Publication Number:** WO 93/22665 **(43) International Publication Date:** 11 November 1993 (11.11.93)

(72) Inventors; and
(75) Inventors/Applicants *(for US only)* : DEMOREST, David, M. [US/US]; 17A Nashua Drive, Scotts Valley, CA 95066 (US). WERNER, William, E. [US/US]; 2760 Melendy Drive, Apartment 1, San Carlos, CA 94070 (US). WIKTOROWICZ, John, E. [US/US]; 6416 San Anselmo Way, San Jose, CA 95119 (US). WENZ, H.-Michael [US/US]; 3733 Country Club Road, Redwood City, CA 94061 (US). OAKS, Frank, L. [US/US]; 3981 Kauri Court, Concord, CA 94521 (US).

(54) Title: CAPILLARY ELECTROPHORESIS MOLECULAR WEIGHT SEPARATION OF BIOMOLECULES USING A POLYMER-CONTAINING SOLUTION
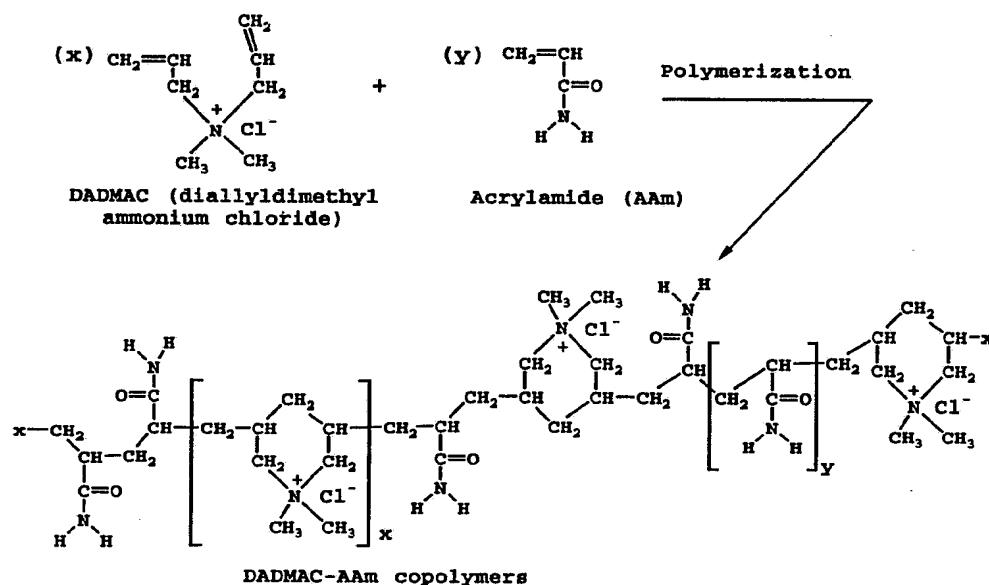
(57) Abstract

A capillary tube with a charged inner surface for use in capillary electrophoresis and methods. The tube is filled with an electrolyte solution containing 0.05-30 % weight/weight of a hydrophilic polymer which is characterized by (A) a molecular weight of 20-5,000 kilodaltons, and (B) a charge between 0.01 and 1.0 % as measured by the molar percent of charged monomer subunits to the total polymer subunits, where said charged monomer subunits have the charge opposite to the wall charge at a selected electrophoresis pH.

1

CAPILLARY ELECTROPHORESIS MOLECULAR WEIGHT SEPARATION OF BIOMOLECULES USING A POLYMER-CONTAINING SOLUTION

Field of the Invention

5   The present invention relates to separation of biomolecules (such as polypeptides, nucleic acids, and oligosaccharides), and in particular, to the use of a low viscosity solution containing a hydrophilic polymer having a charge percent of between about 0.01
10   and 1.0% which is effective as both a molecular sieving matrix and a non-covalent wall coating for capillary electrophoresis.

References

15   Alstine, J.M.V., et al., U.S. Patent No. 4,690,749, issued 9/1/87.

Ausubel, F. M., et al., Current Protocols in Molecular Biology, John Wiley and Sons, Inc., Media PA.

20   Bode, H.-J., Physiol. Chem. Bd. *359S:1237-1238* (1978).

Bohinski, R.C., Modern Concepts in Biochemistry, Second Edition, Allyn and Bacon, Inc.

Cantor, C.R., et al., P.C.T. International
25   Application 83/US1826 111883, WO A1 840524 (1984).

Cantor, C.R., et al., Biochem. 27:9216-9221 (1988).

Cohen, A.S., et al., Anal Chem, *59:1021* (1987).

Cohen, A.S., et al., J. Chromatography, *458:323*
30   (1988).

Cohen, A., et al., U.S. Patent No. 4,997,537, issued 3/5/91.

Compton, S.W., et al. BioTechniques, *6(5):432* (1988).

35   Drossman, H., et al., Anal. Chem., *62:900* (1990).

2

Jen, Yun, Canadian Patent No. 579(222), issued 7 July 1959.

Karger, B.L., et al., European Patent Application No. EP 417925, issued 3/20/91.

5   Kaspar, T.J., et al., J Chromatography, *458:303* (1988).

Kulicke, W.M., et al., Prog Polym. Sci, *8:373* (1982).

Makino, R., et al, PCR Methods and Applications
10   *2(1):10* (1992).

Maniatis, T., et al. <u>Molecular Cloning: A Laboratory Manual</u>, Cold Spring Harbor Laboratory (1982).

Mathew, M.K., et al., Biochem. <u>27</u>:9222-9226
15   (1988).

Mathew, M.K., et al., Biochem. <u>27</u>:9204-9210 (1988).

Mathew, M.K., et al., Biochem. <u>27</u>:9210-9216 (1988).

20   McKormic, C.L., et al., J. Macromol. Sci. Chem. A<u>16</u>(8):1441-1462 (1981).

Mullis, K.B., U. S. Patent No. 4,683,202, issued 28 July 1987.

Mullis, K.B., et al., U. S. Patent No.
25   4,683,195, issued 28 July 1987.

Ogawa, M., U.S. Patent No. 4,657,656, issued 4/14/87.

Ogawa, M., et al., U.S. Patent No. 4,963,243, issued 10/16/90.

30   Takagi, T., et al., Electrophoresis, *12:436-438* (1991).

Wictorowicz, U.S. Patent No. 5,015,350.

Widhalm, A., et al., Chromatographs *549:446-451* (1991).

3

Zhu, D-Z., *et al.*, U.S. Patent No. 5,069,766,
issued December 3, 1991.

Background of the Invention

5   Electrophoresis is widely used for fractionation
of a variety of biomolecules, including DNA species,
proteins, peptides, and derivatized amino acids. One
electrophoretic technique which allows rapid, high-
resolution separation is capillary electrophoresis
10   (CE) (Cohen, 1987, 1988; Compton; Kaspar).
Typically, the CE employs fused silica capillary
tubes whose inner diameters are between about 10-200
microns, and which can range in length between about
5-100 cm or more.

15   In the usual electrophoresis procedure, an
electrophoresis tube, or slab, is filled with a fluid
electrophoresis medium, and the fluid medium is
covalently cross-linked or temperature-solidified to
form a non-flowable, stabilized gel separation
20   medium. A sample volume is drawn into or added to
one end of the tube, and an electric field is placed
across the tube to draw the sample through the
medium. Electrophoretic separation within the matrix
may be based on molecular size, in the cases of
25   denatured protein and nucleic acid species (which
have roughly the same charge density), or on a
combination of size and charge, in the case of
peptides and proteins.

          The polymer concentration and/or degree of
30   cross-linking of the separation medium may be varied
to provide separation of species over a wide range of
molecular weights and charges. For separating
nucleic acid fragments greater than about 1,000
bases, for example, one preferred temperature-
35   solidified material is agarose, where the

4

concentration of the agarose may vary from about
0.3%, for separating fragments in the 5-60 kilobase
size range, up to about 2%, for separating fragments
in the 100-3,000 basepair range (Maniatis). Smaller

5   size fragments, typically less than about 1,000
basepairs, are usually separated in cross-linked
polyacrylamide. The concentration of acrylamide
polymer can range from about 3.5%, for separating
fragments in the 100-1,000 basepair range, up to

10   about 20%, for achieving separation in the size range
10-100 basepairs. For separating proteins, cross-
linked polyacrylamide at concentrations between about
3%-20 percent are generally suitable. In general,
the smaller the molecular species to be fractionated,

15   the higher the concentration of cross-linked polymer.
The resolution obtainable in solidified
electrophoresis media of the type described above has
been limited, in the case of small molecular weight
species, by difficulties in forming a homogeneous,

20   uniform polymer matrix at high polymer concentration
within an electrophoresis tube, and especially within
a capillary tube. In the usual method for forming a
high-concentration solidified matrix in a tube, a
high-concentration monomer solution, (acrylamide and

25   bisacrylamide), is introduced in fluid form fluid
into the tube. The fluid material is then
polymerized, for example, by exposure to light in the
presence of persulfate.
At high polymer concentrations, reaction heat

30   gradients formed within the tube tend to produce
uneven rates of reaction and heat turbulence which
can lead to matrix inhomogeneities. Also, entrapped
gas bubbles generated during the cross-linking
reaction produce voids throughout the matrix. The

35   non-uniformities in the matrix limit the degree of

5

resolution which can be achieved, particularly among
closely related, small molecular weight species.

Alteratively, in the case of temperature-
solidifying polymers the polymer is introduced into
5   an electrophoresis tube in a fluid form, then allowed
to gel to a solid form by cooling. This approach,
however, is generally unsuitable for fractionating
low molecular weight species, such as small peptides
and oligonucleotides, since the polymers, such as
10   agar and agarose, which are known to have the
necessary temperature-solidifying setting properties
are not effective for fractionating low molecular
weight species, even at high polymer concentrations.

A second limitation associated with cross-linked
15   or temperature solidified matrices is the difficulty
in recovering fractionated molecular species within
the matrix, after electrophoretic separation. In the
case of a preparative-scale electrophoresis tube, the
solidified matrix must be carefully separated from
20   the walls of the tube before the matrix can be
removed, a procedure which is virtually impossible
with small diameter tubes. Even after the matrix is
removed, and the region of the matrix containing the
desired molecular species is identified, the species
25   of interest can be recovered from the matrix region
only by a lengthy elution procedure, or by
electrophoretic elution.

In CE, coating materials have typically been
covalently attached to the walls of microcapillary
30   tubes (Cohen, *et al.*, 1991; Karger, *et al.*, 1989;
Alstine, *et al.*, 1987). Most commonly polymerized
matrices are introduced after covalent attachment of
the coating materials (Cohen, *et al.*, 1991; Karger,
et al., 1989). Water soluble polymers have been
35   added to cross-linked, polymerized electrophoresis

6

medium in order to reduce brittleness, i.e., improve
ease of handling, and to improve migration velocity
characteristics (Ogawa, 1987; Ogawa, et al., 1990).

Grossman (U.S. Patent No. 5,126,021, issued 30

5   June 1992) described the use of an uncharged, water-
soluble polymer in a low-viscosity solution which has
a mesh size useful for capillary electrophoretic
separation of biopolymers.

Wictorowicz (U.S. Patent No. 5,015,350)

10   described the use of non-covalent coating to adjust
electroosmotic flow for the separation of
biomolecules. The capillary tube is connected
between anodic and cathodic electrolyte reservoirs,
and an electric field is placed across the reservoirs

15   to produce electroosmotic flow within the tube.
During electroosmotic flow, a compound capable of
altering the surface charge of the tube is drawn into
and through the tube, and the electroosmotic flow
rate within the tube is monitored. The compound is

20   continued to be drawn into and through the tube until
a desired electroosmotic flow rate in the tube, as
determined from said monitoring, is achieved.

Zhu, et al., (U.S. Patent No. 5,069,766)
described the suppression of electroosmotic flow

25   during capillary electrophoresis by the inclusion of
a viscosity raising additive in one or both of the
electrode chamber solutions.

Size-fractionation of protein has been performed
using liquid polyacrylamide (Widhalm, et al., 1991;

30   Takagi, et al., 1991; Bode, 1978). However, protein
separations using liquid polyacrylamide in CE has
attendant problems involving (i) the effect of
electroosmotic flow on protein or protein-complex
migration (Widhalm, et al., 1991), (ii) the use of

35   sufficiently high polyacrylamide concentrations to

attain separation of sample proteins or protein-
complexes into discrete bands (Takagi, et al., 1991),
and (iii) trailing of protein bands (Bode, 1978).

5   **Summary of the Invention**
        The present invention provides a method of
separating biomolecules in a sample. A capillary
tube, having two ends, is prepared. The capillary
tube

10   (i) has charged chemical groups on its inner
wall surface, and
        (ii) is filled with an electrolyte solution
containing 0.05 to 30% weight to weight (w/w) of a
non-cross-linked, hydrophilic polymer or copolymer

15   solution containing at least one polymer or copolymer
species having (a) a molecular weight between 20 and
5,000 kilodaltons, and (b) a percent charge of
between 0.01 to 1.0%. The percent charge is measured
by the molar percent of charged monomer subunits to

20   the total polymer subunits, where the charged monomer
subunits have the charge opposite to the wall charge
at a selected electrophoresis pH. The ends of the
tube are immersed in anodic and cathodic reservoirs
containing an electrolyte solution. The sample,

25   containing the biomolecules to be separated, is
introduced into one end of the tube. An electrical
field is then applied across the reservoirs with a
polarity effective to fractionate said biomolecules
in the sample.

30   Polymers or copolymers useful in the present
invention include the following groups:
polyacrylamides (such as, polyacrylamide, poly(N,N-
dimethylacrylamide) and polymethacrylamide),
polyoxides (such as, polyethyleneoxide and

35 polypropylene oxide), polyethers (such as,

8

polyvinylmethylether), vinyl polymers (such as,
polyvinylpyrollydine, polyvinylalcohol, and
polyvinylacetate), cellulose polymers (such as,
methylcellulose, hydroxyethylcellulose,
5   hydroxylpropylcellulose, and hydroxypropyl-
methylcellulose), acrylic polymers (such as,
polyhydroxyethyl-methacrylate and polyethylene glycol
monomethacrylate), natural gums and polysaccharides
(such as, xanthans, dextrans, agar, guar, and
10   starches).

The polymer or copolymer solution may contain a
homopolymer, a copolymer, or a mixture of the two.

The polymer or copolymer molecules used in the
practice of the present invention contain at least
15   one charged group which has a charge selected from
the group consisting of primary amines, secondary
amines, quaternary amines, carboxylic acids, sulfonic
acids, phosphoric acids, sulfuric acids, and
phosphonic acids. The polymer or copolymer molecules
20   may contain at least one charged group selected from
the group consisting of primary amines, secondary
amines, and quaternary amines, and at least one
charged group selected from the group consisting of
carboxylic acids, sulfonic acids, phosphoric acids,
25   sulfuric acids, and phosphonic acids.

In one embodiment of the present invention the
polymer is a copolymer of acrylamide and diallyldi-
methylammoniumchloride (DADMAC) and the copolymer has
a molecular weight between about 200 and 600 kd.
30   Further, the polymer molecules contain 0.02 to 0.4%
of the quaternary amine N,N-dimethyl-pyrrolidine per
subunit acrylamide.

In another embodiment of the present invention,
the polymer is a copolymer of acrylamide and
35   tetramethylethylenediamine (TEMED), and the copolymer

9

has a molecular weight between about 100 and 500
kilodaltons. Further, the polymer molecules contain
0.05 to 0.5% of the tertiary amine
tetramethylethylenediamine per subunit acrylamide.

5   In another embodiment, the concentration of
polymer molecules containing a charge opposite the
wall charge is sufficient to non-covalently coat the
wall surface and significantly control and reduce
electroosmotic flow (EOF) to less than about 2×10^{-5}
10   cm^2/sec-V.

The method of the present invention may further
include detecting the presence of separated
biomolecules in the electrophoresis capillary tube by
measuring electrochemical, optical (such as UV
15   absorption or fluorescence) or radioisotopic
properties of the biomolecules in the tube.

The present method can be applied to the
separation of proteins, polypeptides, and peptides:
these biomolecules may have a net positive or
20   negative charge at the pH of the electrophoresis
medium. In one embodiment, the biomolecules can be
denatured before separation using, for example,
sodium dodecylsulfate. The denaturant, for example
sodium dodecylsulfate, may also be present in the
25   electrolyte solution.

Further, the present method can be applied to
the separation of nucleic acid fragments. The
nucleic acid fragments can be DNA or RNA, single- or
double-stranded. The differential migration of
30   double-stranded nucleic acids can be adjusted by the
addition of an intercalating agent to the fragments,
to increase preferentially the migration rates of
smaller molecular weight fragments through the
polymer solution. Examples of such intercalating
35   agents are ethidium bromide and acridine orange.

10

The method of the present invention can be applied to
performing restriction digest analysis of a DNA
sample -- after the sample has been treated with one
or more selected restriction endonucleases.

5   The method of the present invention can also be
applied to the separation of linear, branched, native
and chemically modified oligosaccharides.

The present invention further comprises a filled
capillary tube for use in the method of the present

10   invention. The capillary tube has charged chemical
groups on its inner wall surface, and is filled with
an electrolyte solution containing 0.05 to 30% weight
to weight (w/w) of a non-cross-linked, hydrophilic
polymer or copolymer solution containing at least one

15   polymer or copolymer species having (a) a molecular
weight between 20 and 5,000 kilodaltons, and (b) a
percent charge of between 0.01 to 1.0%. As above,
the percent charge is measured by the molar percent
of charged monomer subunits to the total polymer

20   subunits, where the charged monomer subunits have the
charge opposite to the wall charge at a selected
electrophoresis pH.

The polymers and charged groups described above
may be used to fill the capillary tube.

25

Brief Description of the Figures
Figure 1 is a simplified schematic view of a
capillary electrophoresis system used in practicing
the method of the invention.

30   Figure 2 is a schematic view of a capillary
electrophoresis system designed for operation
simultaneously in both a pulsed and constant-voltage
mode;

11

Figure 3 is an enlarged, fragmentary portion of a capillary electrophoresis tube, illustrating electroosmotic flow (e) in a right-to-left direction.

Figure 4 shows the effects on viscosity and EOF
5　of 2% (w/v) polyacrylamide by increasing TEMED concentration in polymerization reactions.

Figure 5 shows the effects on viscosity and EOF of 2% (w/v) polyacrylamide by increasing DADMAC concentration in polymerization reactions.

10　Figure 6 shows the separation into discrete peaks of a protein mixture having multiple components. The copolymer DADMAC/$[AA_m]$ % used for the separation is shown to the right of each electropherogram.

15　Figure 7 shows the separation into discrete peaks of the above-described protein mixture. The copolymer DADMAC/$[AA_m]$ % used for the separation is shown to the right of each electropherogram.

Figure 8 shows a proposed structure for a linear
20　copolymer made from the acrylamide and DADMAC subunits.

Figure 9 shows hypothetical velocity curves for relatively small (dash lines) and relatively large (dash-dot lines) nucleic acid fragments, in relation
25　to an applied square-wave voltage having a pulse width and a maximum voltage v_{max}.

Figure 10 shows the results of a calibration curve for components of a protein mixture relative to the fastest migrating component. The plot shows
30　log(Molecular Weight) versus the relative migration of the protein components.

Figure 11 shows the results of 3 runs sampled from 50 sequential runs of protein separations using the polymer solution of the present invention, where

12

the capillary tube was flushed between runs only with
the polymer solution.

Figure 12 shows the separation of double-
stranded DNA molecules in a cationic copolymer

5   solution of the present invention at 1, 2, and 3%
(w/v).

Figure 13 shows a plot of the log Specific
Viscosity versus the log of Polyacrylamide molecular
weight standards.

10   Figure 14 shows a plot of the log Specific
Viscosity versus the percent concentration of a
single-molecular weight polyacrylamide (367 kD).

Figure 15 shows the plot of relative migration
times of proteins in a protein mixture, relative to

15   the fastest migrating component, versus %
polyacrylamide over a range of % polyacrylamide
values (Ferguson Regression Analysis).

Definitions of Terms

20   The term polymer in the present invention is
used in its traditional sense of a large molecule
composed of smaller monomeric subunits covalently
linked together in a characteristic fashion: i.e., a
homopolymer made of up identical subunits. The term

25   copolymer is used to refer a polymer that contains
two or more kinds of monomeric units in the same
molecule. Through copolymerization there can be made
copolymer materials with different properties than
those of either homopolymer. An example of a

30   copolymer in the present invention is a mixture of
acrylamide and DADMAC to form linear polymer having
the proposed structure shown in Figure 8. In the
specification, a polymer solution refers to a
solution containing a polymer, mixture of polymers,

13

copolymer, mixture of copolymers, or mixture of
polymers and copolymers.

The term hydrophilic describes a
polymer/copolymer which is soluble in water or water
5   buffer systems.

In the context of the present invention the term
biomolecules typically refers to proteins,
polypeptides, peptides, nucleic acids, single and
double stranded DNA and/or RNA. Other biomolecules
10   such as oligosaccharides or glycoproteins may also be
analyzed by the method of the present invention.
Biomolecules may be linear, branched, native or
chemically modified.

Proteins are typically long chains of amino acid
15   based polymers (polypeptides). Proteins may be
composed of one, two or more polypeptide chains and
may further contain some other type of substance in
association with the polypeptide chain(s), such as
iron or carbohydrates. The size of proteins covers a
20   rather wide range from (an arbitrary figure of) 5,000
to several hundred thousand g/mole. The 5,000 figure
corresponds to the presence or roughly 40-45 amino
acids. Proteins smaller than about 5,000 g/mole are
typically referred to as polypeptides or simply
25   peptides (Bohinski).

Percent charge for polymers and/or copolymers in
an electrolyte solution is measured as the molar
percent of charged monomer subunits relative to the
total polymer subunits.

30

Detailed Description of the Invention

The present invention describes the use of at
least one polymer or copolymer in an electrolyte
solution for use in the separation of biomolecules.
35   The polymer containing electrolyte solution (polymer

14

solution) of the present invention is typically
introduced into a capillary tube, where the capillary
tube has charged chemical groups on its inner wall
surface: exemplary of capillaries with negatively
5   charged groups on the inner surface wall are glass or
fused silica. The capillary tube is then filled with
a polymer solution containing 0.05 to 30% weight to
weight (w/w) of a non-cross-linked, hydrophilic
polymer or copolymer: the solution may contain one
10   or more polymer or copolymer species. Typically, the
polymer or copolymer species has (i) a molecular
weight between 20 and 5,000 kilodaltons and (ii) a
percent charge of between 0.01 to 1.0%. At a
selected electrophoresis pH, a charge on the polymer
15   or copolymer is opposite to the charged chemical
groups on the inner wall surface of the capillary
tube.

The ends of the capillary tube are immersed in
anodic and cathodic reservoirs which contain the
20   above-described polymer containing electrolyte
solution. Samples of biomolecules or biomolecule
mixtures are introduced into one end of the capillary
tube and an electric field is applied across the
reservoirs. As the charged biomolecules move through
25   the electric field, they are fractionated on the
basis of size and/or shape by differential migration
through the sieving matrix established by the polymer
solution.

30   I.   Capillary Electrophoresis System
Figure 1 is a simplified schematic view of a
capillary electrophoresis system 20 (Applied
Biosystems, Foster City CA) suitable for practicing
the method of the invention. The system includes a
35   capillary tube 22 having a length preferably between

15

about 10-200 cm, typically less than about 100 cm,
and an inner diameter of preferably between about 10-
200 μm (microns), typically about 50 μm. In the
embodiment shown, the tube is supported in a

5   horizontal position and has downwardly bent end
regions. One preferred capillary tube is a fused
silica tube having an inner diameter of 50 μm and
available from Polymicro Technologies (Phoenix, AZ).
     More generally, the capillary tube may be any

10   tube or channel capable of supporting a column of
polymer solution, preferably at a column internal or
diameter thickness of 200 μm or less. For example,
the tube may take the form of a channel formed in a
glass slide or the like.

15   The inner surface of the capillary tube
typically has charged chemical groups on its inner
wall surface. In one embodiment of the present
invention, the inner surface of the tube has
negatively charged chemical groups at the pH

20   preferably between about 4-9. The surface chemical
groups may be an inherent property of the capillary
material, such as is the case for a fused silica tube
which has surface negatively charged silanol groups.
Alternatively, or in addition, the capillary walls

25   may be treated with known derivatization reagents for
covalent attachment of negative chemical groups, such
as acid groups, to the inner capillary walls, or with
known negatively charged surface-coating agents.
Alternatively, the inner wall surface may be

30   covalently modified to have positively charged groups
covalently attached to the inner wall surface.
Methods for derivatizing or coating glass or the like
are well known in the art. One preferred capillary
tube is a fused silica tube having an inner diameter

16

of 50 μm and available from Polymicro Technologies
(Phoenix, AZ). ·

The polarities indicated below may be switched
depending on the biomolecules to be separated: the
5   detector side reservoir and the sample side reservoir
-- cathodic to anodic or anodic to cathodic. The
polarities of the run, relative to the charge in each
reservoir, can be chosen on many capillary
electrophoresis machines.

10   A cathodic reservoir 26 in the system contains
an electrolytic polymer solution 28; the polymer
solution is described below. The cathodic end of the
tube, indicated at 22a, is immersed in the polymer
solution, as shown, during electrophoresis.

15   A sample tube 30 in the system contains the
biomolecule mixture which is to be loaded into the
cathodic end of the tube. Preferably the sample
material is dissolved in dilute electrolytic solution
or in water. The sample and cathodic reservoirs may
20   be carried on a carousel or the like, for placement
at a position in which the lower cathodic end of the
tube can be immersed in the reservoir fluid.
Although not shown here, the carousel may carry
additional reservoirs containing, for example,
25   solutions for cleaning and flushing the tube between
electrophoretic runs or different polymer solutions.

The opposite, anodic end of the tube, indicated
at 22b, is sealed within an anodic reservoir 32 and
is immersed in an anodic polymer containing
30   electrolyte solution 34 contained in the reservoir,
as shown. The second tube 38 in the reservoir is
connected to a finely-controlled vacuum system (not
shown) for drawing fluid, e.g., washing and cleaning
solutions, electrophoresis polymer solution, through

17

the tube and for loading the biomolecule sample
material in reservoir 30 into the tube. As an
alternative to the vacuum system a positive pressure
system can be used to introduce cleaning solutions,
5   samples, etc.

A high voltage supply 40 in the system is
connected to the cathodic and anodic reservoirs as
shown, for applying a selected electric potential
between the two reservoirs. The power supply leads
10   are connected to platinum electrodes 41, 42 in the
cathodic and anodic reservoirs, respectively. The
power supply may be designed for applying a constant
voltage (DC) across the electrodes, preferably at a
voltage setting of between 5-50 KV. Alternatively,
15   or in addition, the power supply may be designed to
apply a selected-frequency, pulsed voltage between
the reservoirs. In general, the shorter the
capillary tube, the higher the electric field
strength that can be applied, and the more rapid the
20   electrophoretic separation. When operated in a
pulsed voltage mode, the power supply preferably
outputs a square wave pulse at an adjustable
frequency of about 50 Hz up to a KHz range, and an
rms voltage output of about 10-30 KV. Higher pulse
25   frequencies, even into the MHz range may be suitable
for some applications.

Completing the description of the system shown
in Figure 1, a detector 44 in the system is
positioned adjacent the anodic end of the tube, for
30   optically (such as UV absorbance or fluorescence)
monitoring biomolecules migrating through an optical
detection zone 46 in the tube. The detector may be
designed either for UV or visible absorption
detection and/or for fluorescence emission detection
35   or radioisotope detection. UV absorbance is

18

typically carried out at 200-280 range nm, using, for
example, a built-in UV absorbance detector in the
Applied Biosystem Model 270 Capillary Electrophoresis
system, which has a flow cell with a capillary
5   holder.
        Fluorescence emission detection is preferably
carried out at a selected excitation wavelength which
is adjustable between about 240-500 nm, depending on
the fluorescent species associated with the
10 biomolecule, as discussed below. One exemplary
fluorescence detector is an HP1046A detector
available from Hewlett-Packard (Palo Alto, CA), and
modified as above for capillary tube detection. The
detector is connected to an integrator/plotter 45 for
15 recording electrophoretic peaks.
        Radioisotope detection may be accomplished by
the use of a modified HPLC isotope detector for ^{3}H or
^{14}C (Radiomatic Instruments & Chemical Co., Inc.,
Meriden, CT).
20   In operation, the capillary tube is thoroughly
washed by drawing suitable cleaning and rinsing
solutions through the tube by applying a vacuum to
reservoir 32, such as detailed in Example 2. In the
practice of the present invention the polymer-
25 containing electrolyte solution itself can be used to
flush the system between sample runs. If a cleaning
solution different than the polymer-containing
electrolyte solution is used, the tube is then
flushed with several volumes of the electrolytic
30 polymer solution. A small volume, typically 1-10
nanoliters of sample material is loaded into the
cathodic tube end by vacuum injection. A voltage is
applied between the cathodic and anodic reservoirs
until all of the biomolecule peaks have passed
35 through the detection zone.

19

Figure 2 shows a fragmentary view of an
electrophoretic system 50 which can be operated to
the end of the electrophoretic run under a pulsed
field. The capillary tube 52 in the system has a
5   small-clearance break 54 adjacent and upstream of the
detection zone, indicated at 56. The tube sections
on either side of the break are coupled by porous
glass sleeve 58 which allows electrolyte migration
into and out of the tube. The coupled portion of the
10   tube is sealed within a reservoir 60 filled with a
suitable polymer-containing electrolyte solution 62.
A grounded electrode 64 in the reservoir is connected
to the high-voltage side of a pulsed-voltage power
supply 66 whose negative side is in communication
15   with a suitable cathodic reservoir. The grounded
electrode is 64 is connected to the high-voltage side
of a DC power supply 68 whose negative side is in
communication with a suitable anodic reservoir.

In operation, after sample material is loaded
20   into the cathodic end of the tube, the pulsed-voltage
power supply is adjusted to a desired voltage and
frequency level, and the DC power supply to a desired
voltage level. Biomolecules in the sample are
fractionated under the pulsed field within the
25   portion upstream of break 54. Thereafter, the
fragments are carried in a constant-voltage field
through the detection zone, where the fragments can
be optically detected without pulse-frequency noise
effects.

30   Although not shown here, it will also be
appreciated that the electrophoresis system can be
readily adapted for collecting separated biomolecules
for preparative electrophoresis applications. Sample
collection may be accomplished, for example, by

20

providing a series of cathodic reservoirs into which
the fragments can be eluted.

II.   <u>Viscosity Characteristics</u>

5   Viscosity of the polymer or copolymer solution
is a factor in the present invention which determines
the rate at which the solution can be pulled or
pushed through a capillary using the pressures found
on conventional capillary electrophoresis
10   instruments. The rate of solution flow through the
capillary determines how much time is required to
replace the solution matrix between sequential
analyses. An excessive replacement time makes the
current invention less practical or convenient. The
15   flow rate, v, of a fluid with a viscosity, η, moving
through a capillary tube of length, L, and radius, r,
and pressure, p, between the ends is expressed by the
Poiseuille equation:

20                       $v = \pi pr^4/8L\eta$

This equation can be rearranged to calculate
viscosity of a solution that would take time, t, to
replace the entire volume in the capillary tube as:
25

                        $\eta = tpr^2/8L^2$

For example, a capillary that is 50 cm long (L =
50), with a diameter of 50 μM (r = 0.0025 cm),
30   subjected to a pressure of 20" Hg (p = 0.678 Bar),
would require a solution viscosity of less than 38
centipoise (η = 0.38 poise) to have its volume
replaced in less than 30 minutes (t = 1800 seconds),
a time which would be considered excessive for
35   conventional capillary electrophoresis.

21

The viscosity of the polymer/copolymer solution
is determined in the present invention by the
molecular weight of the polymer and its concentration
in the solution. The specific viscosity, η_{sp}, of the
5   solutions is calculated by measuring the time, t, for
the polymer solution, and time, t_0, for water to flow
through a capillary tube at a constant, regulated
pressure and temperature. The ABI Model 270
Capillary Electrophoresis is used as the capillary
10   viscometer in this case with a pressure of 20" Hg and
temperature of 30°C. The specific viscosity is
calculated thus:

$$\eta_{sp} = (t/t_o) - 1$$

15

By using a 2% solution of standard MW linear
polyacrylamides (polyacrylamide, formed by linear
polymerization of acrylamide molecules, is available
in a wide range of molecular weights; Polysciences,
20   Inc., Warrington, PA), a good linear correlation is
shown in Figure 13 between the log of the specific
viscosity (measured as described above) and the log
of the molecular weight. The slope of this
correlation is 1.042 and, in agreement with
25   conventional techniques, this viscosity is a direct
measure of the size of the polymer/copolymer in
solution. Thus, using our example of a viscosity of
less than 38 centipoise (specific viscosity of 30), a
2% polymer solution should use a polyacrylamide of MW
30   less than about 790,000 to avoid excessive
replacement times.
       When the concentration of the polymer or
copolymer is increased in the solution, there is a
corresponding increase in the viscosity of the
35   solution. This fact is shown in Figure 14 where

22

there is a good linear correlation between the log of
the specific viscosity and the % (w/v) of a 367,000
Dalton MW linear polyacrylamide (Polysciences, Inc.)
in solution. So, in the example of a specific
5   viscosity of less than 30, a 367 KD polyacrylamide
should be present in solution at less than about 3.1
% (w/v) to avoid excess replacement times.
    The relationship between viscosity and
concentration of the polymer/copolymer suggests that
10   if high concentration is required to obtain
resolution of biomolecules in a mixture, then a lower
molecular weight polymer/copolymer can be used to
keep the viscosity low. Conversely, at lower percent
concentrations of polymer/copolymer higher molecular
15   weight polymer/copolymers can be used.
    The MW of the polymer or copolymer is adjusted
by a number of different methods. First, the
conditions of polymerization are changed in order to
effect changes in the MW of the final polymer
20   product. The viscosity average MW of the
copolymer/polymer is decreased by (1) increasing the
reaction temperature, (2) increasing the content of a
water miscible solvent in the reaction mixture, such
as methanol, or (3) increasing initiator
25   concentrations. The above list of reaction
conditions is not inclusive, as other polymerization
conditions or additives are used to control MW,
dependent on the particular polymer/copolymer that is
made.
30   A second method of adjusting the average MW of a
polymer/copolymer is by fractionating a polydisperse
polymer product into different MW fractions followed
by isolation and purification. An aqueous solution
of polymer/copolymer is fractionated by (1) a size-
35   dependent chromatographic separation (such as gel

23

permeation chromatography), (2) a dialysis using
membranes of specified MW cut-offs, or (3) fractional
precipitations using a water miscible solvent such as
methanol.

5   The concentration of the polymer/copolymer
solution is adjusted by (1) adding different weights
of solid polymer to a specified volume of aqueous
buffer and mixing until the solid is completely
dissolved or (2) adding different weights or volumes
10   of a concentrated aqueous polymer solution to a
specified volume of aqueous buffer and mixing until
the concentrate is completely dispersed.

          It is clear that the upper limits of polymer/-
copolymer MW and/or its concentration in solution
15   will be dictated by primarily the upper viscosity
that can be pushed or pulled through a capillary.
This upper viscosity is set by the instrumental
parameters as expressed in the previous equations.
Thus, for example, if the capillary electrophoresis
20   used a short capillary (L = 20 cm) with a large
radius (r = 0.01 cm), a solution with a viscosity of
about 38625 centipoise could be pushed through the
capillary in 30 minutes at high pressure (P = 100 psi
= 6.87 bar). Using the data of Figure 13 (log
25   Specific Viscosity vs. Polyacrylamide concentration),
it can be estimated that the upper concentration is
about 9% (w/v) for a polyacrylamide of MW = 367 KD.
Obviously, by utilizing a polyacrylamide of lower MW,
solutions for higher concentrations could be pushed
30   through a capillary and it is not unreasonable to
expect that concentrations approaching 20% (w/v)
would be useable.

          As is clear from the relationships set forth
above, an instrument that has higher pressure can
35   give a higher flow rate for a given concentration of

24

polymer relative to an instrument which generates a
lower pressure. As a further example, a larger
diameter capillary can give a higher flow rate
relative to a smaller diameter capillary tube. The
5   factors affecting flow rate can be manipulated based
on the parameters defined above which affect flow
rate.

III. Polymer Solutions and Electroosmotic Flow
10   The phenomenon of electroosmotic flow in a
polymer solution matrix is described for an inner
capillary tube wall having net negative charge with
reference to Figure 3, which shows an enlarged,
fragmentary portion of a capillary electrophoresis
15   tube 70.
As seen in Figure 3, the negatively charged
groups on the inner tube wall, indicated by "-"
symbols, are shielded by both loosely and tightly
bound positively charged ions present in the polymer
20   electrolyte solution, essentially forming a
positively charged shell about the column of fluid in
the tube. The thickness of the shell of positive
ions at the wall surface is known as the electrical
double layer. The electric double layer is
25   characterized by a zeta potential, which is a measure
of the potential at the capillary wall.
Under the influence of an electric field, this
column of polymer solution in the medium (which is
surrounded by a shell of positive charges) is drawn
30   electroosmotically in the direction of movement of
the positive shell, (i.e., towards the cathode). The
rate of electroosmotic flow in the tube is indicated
by the arrow e in the figure (arrow e may be thought
of as a vector with a magnitude e and a direction
35   along the axis of the tube). The electroosmotic flow

25

rate e in a capillary tube can be described by the
equation:

$$e = \epsilon \zeta E / 4\pi\eta$$

5

where ϵ, η, ζ, and E are the dielectric constant of
the double layer, its viscosity; the zeta potential;
and the electrical field strength, respectively.

In the method of the present invention, the
10   polymers or copolymers in the electrolyte solution
have a sufficient percent charge to bind to the
oppositely charged capillary wall. This binding
significantly reduces or eliminates electroosmotic
flow in the capillary tube by decreasing the
15   dielectric constant, or increasing the viscosity, or
both, of the electrical double layer. The level of
electroosmotic flow must be reduced sufficiently to
allow for discrete peaks or bands of separated
biomolecules without substantial tailing, or
20   broadening, of the bands, i.e., decreased resolution.
As mentioned above, separation of biomolecules by the
method of the present invention, relies predominantly
on the sieving effect provided by the polymers and/or
copolymers in the electrolyte solution. The effect
25   of the percent charge in a particular
polymer/copolymer solution on electroosmotic flow can
be evaluated as follows.

Electroosmotic flow is typically measured as the
mobility (cm^2/sec-V) of an electrically neutral
30   substance through a capillary tube containing a
defined polymer/copolymer electrolyte solution
(Example 2).

IV.   Separation of Biomolecules

26

The separation method of the present invention
can employ a number of polymers and copolymers in
formulation of the polymer-containing electrolyte
solution.

5   Typically, the polymers or copolymers used in
the method of the present invention have a molecular
weight of between 20 and 5,000 kilodaltons.
Exemplary polymers and copolymers useful in the
present method including the following:
10   polyacrylamides, such as polyacrylamide, poly(N,N-
dimethylacrylamide) and polymethacrylamide;
polyoxides, such as polyethyleneoxide and
polypropylene oxide; polyethers, such as
polyvinylmethylether; vinyl polymers, such as
15   polyvinylpyrollydine, polyvinylalcohol, and
polyvinylacetate; natural gums or polysaccharides,
such as xanthans, dextrans, agar, guar and starches;
cellulose polymers, such as methylcellulose,
hydroxyethylcellulose, hydroxylpropylcellulose, and
20   hydroxypropyl-methylcellulose; and acrylic polymers,
such as polyhydroxyethylmethacrylate and polyethylene
glycol monomethacrylate. Mixtures of polymers and
copolymers can also be used in the practice of the
present invention.
25   The percent charge of a selected polymer or
copolymer can be achieved in a number of ways
(Example 1).   When homopolymers are used in the
method of the present invention they can be modified
to contain a specified percent charge. After or
30   during polymerization, a homopolymer may be modified
to contain a desired average percent charge, such as
applying a Hoffman degradation to polyacrylamide to
produce vinylamines (Kulicke). An example of a
homopolymer is polyacrylamide, available in a wide
35   range of molecular weights, linearly polymerized in

the presence of ammonium persulfate to form a
polyacrylamide molecule.

Copolymers are also useful in the method of the
present invention. One advantage of copolymers is
that one of the subunits may be specifically chosen
to contain a desired charge group. This subunit can
then be added to polymerization reactions with
another subunit at a defined concentration thereby
allowing formation of a copolymer having the desired
percent charge characteristics. For example,
copolymers of acrylamide ($[AA_m]$) and
tetramethylethylenediamine (TEMED), or acrylamide and
diallyldimethylammoniumchloride (DADMAC) can be
formed as described in Examples 1 and 2. These
copolymers can cover a wide range of percent charge
values, as well as a range of viscosity values.

By substituting other types of acrylamides in
the reaction schemes of Examples 1 and 2, other types
of charged copolymers can be synthesized, such as
poly(methacrylamide), poly(N-methylacrylamide) or
poly(N,N-dimethylacrylamide). For example, the
polymer can be a copolymer of N,N-dimethylacrylamide
and tetramethylethylenediamine (TEMED), where the
copolymer has a molecular weight between about 200
and 800 kilodaltons, and the polymer molecules
contain 0.03 to 0.6% of the tertiary amine
tetramethylethylenediamine per subunit of N,N-
dimethylacrylamide.

Further, graft or block copolymers are also
useful in the practice of the present invention.
Aqueous graft charged polymers can be formed by
polymerization of charged copolymers in the presence
of uncharged polymers. One example of such a
grafting reaction is presented in Example 1D for the

28

grafting copolymer of acrylamide and DADMAC onto
dextran.

Charged groups or subunits useful in
modification of the polymers and copolymers of the
5   present invention include the following: primary
amines, such as vinylamine, glucosamine; secondary
amines, such as ethylenimine; tertiary amines, such
as vinyl pyridine, and tetramethylethylenediamine
(TEMED); quaternary amines, such as vinyl-N-
10   methylpyridine, N,N-dimethyl-3,5-methylene piperidine
(DADMAC); carboxylic acids, such as acrylic acid,
methacrylic acid, and malic acid; sulfonic acids,
such as vinyl sulfonic acid; phosphoric acids, such
as vinyl phosphoric; sulfuric acids, such as vinyl
15   sulfuric; and phosphonic acids, such as
vinylphosphonic.

By substituting other types of charged subunits
for DADMAC, in the reaction scheme of Example 2,
other types of charged copolymers can be synthesized,
20   such as a polyacrylamide that contains a low per cent
of the charged subunit derived from the following
compounds: [3-(methacrylolamino)propyl]trimethyl-
ammonium chloride or the subunit 2-methacryloxyethyl-
(trimethylammonium) chloride. Similarly, the TEMED
25   in Example 1 can be replaced by an equally reactive
compound that would introduce a charged group into
the polyacrylamide, such as 2,2'-azobis(2-
methylpropionamidine) dichloride.

For example, the polymer can be a copolymer of
30   acrylamide and [3-(methacryloylamino)propyl]tri-
methylammonium chloride, where the copolymer has a
molecular weight between about 200 and 600
kilodaltons, and the polymer molecules contain 0.01
to 0.2% of the quaternary amine N-[(propyl)trimethyl-

29

ammonium chloride]methacrylamide per subunit of
acrylamide.

The above-listed molecules containing charged
5   groups can be incorporated in homopolymers by methods
known in the art (see Example 1). Polymers and
copolymers useful in the present invention may also
be ampholytic polymers which contain both positive
and negative charge groups: the different charged
10   units can be introduced into the same polymer,
including mixes of positive and negative charge
groups. For example, the polymer or copolymer
molecules may contain at least primary amine,
secondary amine, and/or quaternary amine group, and
15   at least carboxylic acid, sulfonic acid, phosphoric
acid, sulfuric acid, and/or phosphonic acid group.
Use of such ampholytic polymers may be useful for
separations since, for example, the positive charge
on the polymer can bond non-covalently with the
20   negative capillary wall, and the slight negative
charge on the polymer may improve separation by
charge repulsion. In solution, the positive and
negative charges on the polymer may also bond non-
covalently and modify the sieving properties of the
25   matrix.
In several cases, the above-listed charged
group-containing molecules can also be directly
incorporated into co-polymers as, for example, is
illustrated in Example 1 for acrylamide and DADMAC.
30   Example 2 describes the use of copolymers of
acrylamide ($[AA_m]$) and TEMED or DADMAC. The rate of
electroosmotic flow was measured using a neutral
marker. The proportions of TEMED or DADMAC to $[AA_m]$
were varied over the ranges of values shown at the
35   bottom of Figures 4 and 5. In the cases of both

30

TEMED and DADMAC, the rate of electroosmotic flow
(EOF) was significantly reduced (less than about 2×10^{-5} cm^2/sec-V) at percent charge values of 0.05%.

Figure 4 shows the effects on viscosity and EOF

5   of increasing TEMED concentration in polymerization
reactions. Using a constant polyacrylamide
concentration of 2% (w/w) to measure EOF, the EOF
decreased with increasing % TEMED and the results
presented in Figure 4 show that the EOF remains below

10   2×10^{-5} cm^2/sec-V for percent charge values covering
the range of approximately 0.05% to 0.5%. The
viscosity of the polymer solution decreased with
increasing % TEMED and remained relatively constant
above about 0.2%. These data demonstrate that

15   increases in the percent positive charge and not
increases in viscosity, result in a decrease in EOF.

Figure 5 shows the effects on viscosity and EOF
of 2% (w/w) polyacrylamide made with increasing
DADMAC concentration in polymerization reactions. As

20   can be seen from the results presented in Figure 5,
EOF decreases with increasing % DADMAC and remains
below 2×10^{-5} cm^2/sec-V for percent charge values
covering the range of approximately 0.05% to 0.26%.
The viscosity of the polymer solution increases and

25   decreases with increasing % DADMAC but increases in a
linear fashion in the same range of .05 to .26%.
DADMAC/[AA$_m$] copolymers having percent charge values
in the range of 0.05% to 0.5% are useful for the
separation of a wide variety of biomolecules having

30   different size and/or shape. Thus, this data also
shows that the decrease in EOF is due to increasing %
of positive charge and not due to increasing
viscosity.

The procedure described in Example 2 is useful

35   for the control of percent charge, contained in

31

polymers or copolymers to be used in the practice of
the method of the present invention, in order to
obtain a reduced EOF.

For the separation of biomolecules the capillary
5   tube is typically filled with an electrolyte solution
containing 0.05 to 30% weight to weight (w/w) of a
non-cross-linked, hydrophilic polymer or copolymer
solution which contains at least one polymer or
copolymer species having (a) a molecular weight
10   between 20 and 5,000 kilodaltons, and (b) a percent
charge of between 0.01 to 1.0% as measured by the
molar percent of charged monomer subunits to the
total polymer subunits. A charge on the polymer or
copolymer in solution is opposite to the wall charge
15   at a selected electrophoresis pH.

The results presented in Figures 4 and 5
demonstrate that it is not viscosity that is
responsible for the reduction of EOF, rather it is
the percent charge of the polymer/copolymer molecule.
20

Example 3 describes the use of a number of
copolymer solutions for the separation of
biomolecules. The separation of a mixture of
proteins was used to illustrate the molecular sieving
25   capabilities of the present method. Figure 6 shows
the separation into discrete peaks of a protein
mixture having the following components: α-
lactalbumin (14 kd), trypsin inhibitor (20 kd), egg
albumin (45 kd), bovine albumin (66 kd), and β-
30   galactosidase (116 kd). In Figure 6 the order of the
peaks from left to right corresponds, respectively,
to the just-listed proteins. The DADMAC/[AA$_m$] % used
for the separation is shown to the right of each
electropherogram. As can be seen from Figure 6,
35   percent charge values in the range of 0.26, 0.1, to

32

0.05% are all effective for the separation of each
protein in the protein mixture. Comparison of the
separation ability of the copolymer electrolyte
solution (Figure 6) to the EOF of the same copolymer
5   solution (Figure 5) shows that each of the values
0.26, 0.1, and 0.05% have an EOF mobility value of
less than 2×10^{-5} cm^2/sec-V.

At the 0.26% value, significant baseline,
downward sloping occurs which would obscure data at
10   the longer run times. In general, the upper limit of
percent charge is determined as that value which does
not cause significant baseline perturbations (i.e.,
sloping, spiking, etc.). Although it is dependent on
the polymer type, this upper value rarely exceeds 1%.

15   Figure 7 shows the separation into discrete
peaks of the above-described protein mixture. The
DADMAC/[AA$_m$] % used for the separation is shown to
the right of each electropherogram. As can be seen
from Figure 7, percent charge values of 0.02% and
20   less are ineffective for the separation of each
protein in the protein mixture because of significant
band-broadening and loss of sensitivity. Comparison
of the separation ability of the copolymer
electrolyte solution (Figure 7) to the EOF of the
25   same copolymer solution (Figure 5) shows that each of
the values 0.02% and less have an EOF mobility value
of greater than 2×10^{-5} cm^2/sec-V.

In addition to the separation of components of a
mixture of biomolecules, the method of the present
30   invention is also useful for molecular weight
determinations of a selected biomolecule. Example 3B
describes the generation of a molecular weight
calibration curve based on the relative migration of
known protein standards to a selected molecular
35   weight standard. Figure 10 shows the calibration

33

curve of log(MW) versus the migration of the protein
standards relative to α-lactalbumin (the selected
reference standard). Any number of compounds having
a known molecular weight and charge can be used as
5   the reference standard to establish relative
migration: for example, charged organic molecules;
dyes; nucleic acids; proteins, and other charged
biomolecules. In practice, the migration of the
compound having unknown molecular weight is
10   determined relative to the selected reference
standard, such as α-lactalbumin, and the molecular
weight is then determined by comparison to a standard
curve.

Example 3C demonstrates the reproducibility of
15   the method of the present invention. A 3% w/v
acrylamide/TEDMED copolymer solution was used for the
separation of a mixture of protein standards. Fifty
sequential runs were performed using the same mixture
of proteins. Between each run the capillary was
20   flushed using the acrylamide/TEMED copolymer
solution. The separation results are shown in Figure
11 for runs 1, 30 and 50. As can be seen from Figure
11, separation of the components of the protein
mixture is very reproducible by the method of the
25   present invention.

In Example 3D the effect of changing the percent
concentration of polymer/copolymer in the solution on
the relative migrations of biomolecules was examined.
As the percent concentration of the polymer/copolymer
30   changes, the differences in relative migration
between the protein standards changes as well (Figure
15). The slope of the lines in Figure 15 for each
protein standard are proportional to molecular
weight, indicating that the polymer/copolymer
35   solution is providing a true sieving matrix. Also,

34

this method is another way to determine molecular
weight for a given biomolecule: compare the slope of
the line for an unknown biomolecule to a plot of the
slopes of the lines of the molecular weight
5   standards. The relative migration in Figure 15 is
the rate of migration of a particular protein
relative to the migration of α-lactalbumin, i.e., the
migration time of α-lactalbumin divided by the
migration time of each standard protein.

10   Example 4 demonstrates the separation of DNA
fragments in a mixture of restriction digest
fragments. φX174 RF *HaeIII* digest fragments were
separated by the method of the present invention
using polyacrylamide made with TEMED at 0.24 percent
15   charge. There are 11 φX174 RF *HaeIII* digest
fragments ranging in size from 72 to 1353 base pairs.
Figure 12 shows the resulting electropherograms for
three copolymer concentrations 1, 2 and 3%. The data
in Figure 12 illustrates the property of the method
20   of the present invention that increasing the percent
concentration of the polymer/copolymer affects the
separation ability of the technique. As can be seen
in Figure 12, as copolymer concentration increases
the resolution of the peaks corresponding to the
25   φX174 RF *HaeIII* 271 and 281 base pair fragments
improves: at 2% they are starting to separate and at
3% percent the peaks are well resolved. This example
illustrates how manipulating the percent
concentration of the polymer in solution affects
30   resolution.

V.   Optimization of Separation Conditions
The characteristics of polymers useful in the
present method for the adjustment of electroosmotic

35

flow and molecular sieving in capillary
electrophoresis include the following:

    1.   an electrolyte solution containing 0.05 to
30% weight to weight (w/w) of a non-cross-linked,
hydrophilic polymer or copolymer solution containing
at least one polymer or copolymer species;

    2.   where the polymer or copolymer species has
a molecular weight between 20 and 5,000 kilodaltons;
and

    3.   where the polymer or copolymer species has
a percent charge of between 0.01 to 1.0% as measured
by the molar percent of charged monomer subunits to
the total polymer subunits, where the charged monomer
subunits have the charge opposite to the wall charge
at a selected electrophoresis pH.

    For any selected polymer, copolymer, or mixture
thereof, the percent charge value can be optimized by
selecting a range of percent charge values
essentially as described in Example 2 to discover the
value for which electroosmotic flow is preferentially
reduced to less than 2×10^{-5}. A further test of a
selected polymer or copolymer solution is the
separation of a target biomolecule sample. The %
(w/w) polymer/copolymer in solution should be
adjusted to obtain separation of the components of
the selected mixture of biomolecules (for example, as
shown with the above-described protein or DNA
mixture). The solution should resolve components on
the basis of size of the target biomolecule.

    In addition to the use of polymers or copolymers
bearing specific % charges, or in solutions at
selected % (w/w) the rates of fragment migration may
be selectively adjusted by:

    1.   Changes in the electric field strength.
Migration times and to some degree resolution may be

36

adjusted by performing the separation at about 100 to
400 V/cm.

     2.   Changes in the solution pH. Biomolecules,
particularly proteins, may assume different net
5   charge at different pH values. By adjusting the pH
of the polymer solution between about pH 4 to 10, the
relative migration rate may be altered.

     3.   Changes in temperature. Migration times
and resolution may be changed by performing
10   separations at temperatures of about 10 to 60°C.
Lower temperature generally improves resolution.

     4.   Changes in buffer type concentration or
ionic strength. For any given buffer species used in
the polymer/copolymer solution, there exists an
15   optimum range of concentration for maximum
resolution. Band-broadening may occur at a too low
concentration (low ionic strength) or band-broadening
may occur at too high of a ionic strength. In
particular, zwitterionic buffers improve resolution
20   and reduce background UV absorbance variations.

     5.   Addition of surfactants to buffer. The
incorporation of hydro- or fluorocarbon surfactants
with neutral, positive, or negatively charged head
groups will selectively alter migrations of proteins.
25   In particular, the presence of sodium dodecylsulfate
(SDS) in buffer results in migrations proportional to
the protein molecular weight.

     6.   Addition of denaturants to the buffer.
Altered migrations of single- or double-stranded DNA
30   and improvement in resolutions may be effected by
addition of denaturants such as urea or formamide to
the electrolyte buffer.

    Summarizing the above, the present invention
provides a variety of parameters which may be
35   selectively varied to enhance biomolecule

37

fractionation. The biomolecule migration rates can
be selectively altered by changing the nature of the
polymer and its concentration, the pH of solution,
separating temperature and field strength, and buffer
5   composition. For nucleic acids, the migration rates
can be selectively changed by complexing the
fragments with a non-ionic intercalating agent, such
as ethidium bromide or acridine orange, and
resolution can be improved by addition of denaturants
10   such as urea and formamide.

        VI.   Pulsed Field Separation
                The electrophoretic methods described above were
        carried out under a constant-voltage field.   In
15   accordance with another aspect of the invention, the
        fractionation of biomolecules, in particular, nucleic
        acid fragments, can be enhanced by carrying out the
        electrophoretic separation under a pulsed-voltage
        field, at a frequency effective to selectively
20   enhance separation within a given fragment size
        range.·
                In theory, the migration rate behavior of
        nucleic acid fragments in a pulsed field may be
        governed by two size-related effects.   The first
25   effect is a resonance effect involving the fragment's
        rotational modes and the frequency of the electric
        field.   Table 1 below shows rotational and stretching
        resonance frequencies which have been calculated for
        100, 1,000, and 10,000 basepair duplex DNA fragments.
30   The rotational resonance frequencies in Hz were
        calculated on the basis of a prolate ellipsoid model
        of the duplex molecule (Mathew, et al.; Cantor, et
        al.).

38

Table 1

| Model | Fragment Size (base pairs) | | |
	100	1000	10,000
Prolate Elipsoid (rotational motion)	9.9×10^4	1.6×10^2	2.2×10^{-1}
Viscoelastic (stretching motion)	3.8×10^5	8.1×10^3	1.7×10^2

A strong rotational resonance effect predicts
that the migration rates of fragments in resonance
with the electric field will be preferentially slowed
with respect to the migration rate in a time-
invariant field. This is because a molecule in rota-
tional resonance with the electric field would be
expected to be, on average, least favorably oriented
for migration in the direction of the field when the
electric field is greatest. Larger molecules,
because of their slower rotational times, would be
expected to be less perturbed from their field-
oriented positions at each voltage-pulse cycle;
smaller molecules, with their faster response times,
would more quickly reorient in the direction of the
field. Thus, if rotational resonance effects are
dominant, it should be possible to slow the migration
of resonance species during electrophoresis relative
to the rate of migration of non-resonance species.

The second size-dependent effect which may be
expected in a pulsed field is an inertial effect due
to the acceleration and deceleration of fragments in
a fluid with each voltage pulse. This effect is
illustrated in Figure 9, which shows hypothetical
velocity curves for relatively small (dash lines) and
relatively large (dash-dot lines) nucleic acid
fragments, in relation to an applied square-wave

voltage having a pulse width and a maximum voltage
V_{max}. The maximum velocity which the fragments should
reach is the terminal or steady-state velocity of the
fragments in a constant voltage field of potential

5   V_{max}, i.e., 100% of the constant field migration rate.

As seen from the figure, smaller fragments are
expected to reach terminal velocity more rapidly than
large ones after application of the voltage pulse,

10   but to decelerate to zero voltage substantially at
the same rate when the voltage pulse ends. Since the
total distance traveled by each fragment during a
voltage pulse is just the integral of the velocity
curve, the migration rates of larger fragments are

15   expected to be preferentially decreased in a pulse-
voltage field. It can also be appreciated that the
higher the pulse frequency and shorter pulse dura-
tion, the smaller the fragment size which can be
preferentially retarded in its migration rate, since

20   the effect of any lag in the velocity curve becomes
accentuated at short pulses.
In some mixtures of biomolecules, in particular
nucleic acid fragments where it is desired to
fractionate species in different size ranges, the

25   variables discussed above -- including solution pH,
and polymer type and concentration, field frequency,
and denaturant -- may be selectively varied during
the electrophoretic run to enhance fragment
resolution. For example, an electrophoretic

30   separation may be carried out under a constant field
or low frequency initially, to resolve larger size
fragment, then switched to a higher frequency to
improve resolution of smaller size fragments. As
another example, the pH or polymer concentration of

35   the polymer solution can be continuously varied

40

during an electrophoretic run, using a standard two-chamber mixing device to produce a continuous solution gradient which is drawn into the capillary tube.

5

        VII. Utility
        The fractionation method of the invention finds utility in any of a variety of applications mentioned above requiring size fractionation of biomolecules.
10   These applications include electrophoretic separations of proteins, polypeptides, peptides and single and double strand nucleic acids, including restriction analysis of DNA.
        The method of the present invention can be
15   applied to DNA sequencing analysis where the DNA sequencing reactions contain nucleic acids labelled with fluorescent tags (Drossman) and the products of these reactions are size fractionated by the method of the present invention. Nucleic acid molecules can
20   be size-fractionated under either non-denaturing or denaturing conditions. Typical denaturing conditions include the addition of urea or formamide to the nucleic acids before separation and/or to the electrolyte solution.
25   Further, the method of the present invention can be used in single-stranded conformation polymorphism analysis of DNA (Makino). Polymerase chain reaction methods (Mullis; Mullis, et al.; Perkin-Elmer Cetus Corp.) can also be applied to single-strand
30   conformation polymorphism analysis. This analysis is based on the principle that single strand DNA molecules develop conformations that are sequence specific and that are stabilized by intrastrand interaction: typically such analysis is carried out
35   under non-denaturing conditions, i.e., conditions

41

that maintain conformation features of the single-
strand molecules. Even a single base change between
single-stranded molecules can alter the conformation
enough to detect a relative mobility difference
5   between the molecules. The conditions under which
size-fractionation of single-strand DNA molecules is
carried out can be varied to affect mobility of the
single strand molecules. For example, the following
parameters may be varied: % polymer, type of
10   polymer, glycerol (typically present at a concen-
tration of about 5% to 20%, w/v), and temperature.
        The fractionation method of the present
invention is also useful for the analysis of
polymerase chain reaction amplification products.
15   For example, amplification reactions can be carried
out using a sample template and selected primers.
The products are size-fractionated to determine if
the target amplification product, having an
anticipated size, is present in the reaction mixture.
20   The concentration of amplification reaction products
can also be determined.
        The fractionation method of the invention can
also be used in the analysis of nucleic acid-protein
complexes. For example, the method can be used in a
25   migration-shift assay format, where the ability of a
sample protein to bind a polynucleotides that
contains a selected nucleotide sequence is assessed
by comparison of the mobility of the polynucleotide
in the presence and absence of the sample protein.
30   Binding of the polynucleotide to the sample protein
can be confirmed when the mobility of the resultant
polynucleotide-protein complex is different from that
of the polynucleotide alone. The method may also be
used to determine the stoichiometry of binding
35   between the sample protein and the polynucleotide by

42

varying the ratio of sample protein to polynucleo-
tide. Conveniently, the polynucleotide contains a
distinctive reporter label, such as a fluorescent
tag, which allows polynucleotide-containing peaks to
5 be easily identified and quantitated.

Applications of the analysis of restriction
digestion products include the following: analysis
of restriction fragment length polymorphisms for
genetic screening, confirming vector construction,
10 identifying specific nucleic acid fragments on the
basis of size and/or hybridization to nucleic acid
probes, and fractionating single-strand fragments for
chemical or enzymatic sequencing.

The following applications illustrate how the
15 method may be used for restriction fragment analysis.
In the restriction analysis example, it is desired to
identify, from a mixture of genomic fragments, a
restriction fragment which contains a target sequence
of interest. After digesting the genomic mixture
20 with a selected restriction enzyme or enzyme(s), the
fragment mixture is combined under hybridization
conditions with reporter-labelled probe capable of
hybridizing with the target sequence. The probe
preferably includes the complementary target sequence
25 and a covalently bound probe, such as a fluorescent
probe which can be readily detected by a fluorescence
probe detector. The probe may be hybridized with the
fragments, for example, by standard
denaturation/renaturation conditions involving
30 single-strand species, or bound to the fragments in
duplex form by RecA catalyzed triplex formation.

After binding the probe to the fragments, the
sample is fractionated according to present method.
The detector is preferably operated in a dual-
35 wavelength mode, in which UV absorption and

fluorescence emission detection are carried out
concurrently.

By the method of the present invention,
proteins, polypeptides, and peptides, either in a
5   denatured state, native state, or chemically
modified, are separated according to their size or
molecular weight by electrophoresis in a tube
containing a polymer and/or copolymer electrolyte
solution. When this solution is employed for
10   separation by capillary electrophoresis, very rapid
and high resolution molecular weight (MW) separations
of SDS (sodium dodecylsulfate) denatured proteins are
obtained (see Figures 6 and 7). By correlating
relative migration times with molecular weights of
15   standard proteins, the molecular weight of unknown
proteins can be estimated.

In addition to the separation of proteins and
nucleic acids, the method of the present invention
can also be applied to the size-fractionation of
20   nucleic acid/protein complexes, such as
ribonucleoprotein particles, DNA-binding proteins
bound to their cognate DNAs, and RNA binding proteins
bound to their cognate RNA-binding sites (such as the
TAT and REV proteins of HIV-1).

25   High mass sensitivity and reproducible migration
times are obtained by utilizing polymers or
copolymers which have a percent charge in the range
of approximately 0.01 to 1.0 %, where the charge on
the polymers or copolymers is opposite the charge on
30   the inner surface of the capillary tubing. This
combination serves to substantially reduce
electroosmotic flow to below approximately 2×10^{-5}
$cm^2/sec-V$ and to prevent adsorption of proteins
during electrophoresis. Not all applications require
35   reduction of electroosmotic flow to below 2×10^{-5}

44

$cm^2/sec-V$:   substantial reduction of electroosmotic
flow occurs at approximately 8×10^{-5} $cm^2/sec-V$.   The
polymer solution used for separation is introduced
into the capillary tube using moderate pressures

5   found in conventional capillary electrophoresis
equipment.

After separation of a sample containing
biomolecules, the capillary is flushed with fresh
polymer solution before analysis of the next sample.

10 This step eliminates the possibility, when processing
sequential samples, of contamination, or "ghost
peaks" from previous samples.   The ability to flush
the system with fresh polymer solution is made
possible by limiting the viscosity of the polymer

15 solution.

The polymer or copolymer used in the separation
solution has a molecular weight within the range of
20 and 5,000 kilodaltons.   The molecular weight of
this polymer is typically above a threshold value for

20 entanglement which will exert a retardation to
electrophoretic migration of the selected
biomolecules, which is proportional to the molecular
weight of the biomolecules (Grossman, U.S. Patent No.
5,126,021, issued 30 June 1992).   The molecular

25 weight of the polymer or copolymer in solution is
below a value which results in a solution of high
viscosity that cannot be introduced into a narrow
capillary (less than 200 μM I.D.) using pressures
found in conventional capillary electrophoresis

30 equipment.

The polymer or copolymer electrolyte solution
may also contain an organic or inorganic ion, used
for pH control and optical stability, including the
following: Organic acids (such as citric, acetic,

35 formic) or zwitterionics (such as TES (N-

45

tris[Hydroxymethyl]-2-aminoethanesulfonic acid)
(Sigma), BICINE (N,N-bis[2-hydroxyethyl]glycine)
(Sigma), ACES (2-[2-Amino-2-oxoethyl)-
amino]ethanesulfonic acid) (Sigma)), glycylglycine
5   (Sigma); Inorganic acids (such as phosphoric); and
Organic bases (such as "TRIS" (Sigma)).
        When performing denaturing separations, an
anionic surfactant can also be added to the polymer
or copolymer electrolyte solution. The anionic
10   surfactant is typically a hydrocarbon or fluorocarbon
sulfate (such as sodium dodecylsulfate (SDS)),
sulfonic (such as sodium decanesulfonic), or a
carboxylic (such as lauric acid). For example, an
SDS denatured protein sample, diluted in water, can
15   be injected electrokinetically into the capillary.
Separations are usually completed in under 15 minutes
when using about a 30 cm separation length and 200
V/cm field strength.
        When employing on-column UV detection at
20   wavelengths less than 240 nm, the zwitterionic
buffers such as, ACES or TES (Sigma), in a separation
solution containing polyacrylamide and SDS at pH 6 to
8, exerts a stabilization effect on the background UV
absorbance. This stabilization permits repeatable
25   and sensitive UV detection at 200 nm without
excessive baseline sloping.
        The use of a polymer or copolymer solution as
described above for the capillary electrophoretic
separation of proteins or nucleic acids by molecular
30   weight is an advantage over conventional PAGE slab
gel separations by providing (1) a shorter analysis
time, (2) a totally automated separation and
detection, (3) a quantitative analysis, (4) a non-
destructive analysis of extremely small amounts of
35   proteins or nucleic acids (picograms), and (5) a user

46

convenient matrix (i.e., a pre-tested polymer
solution and no solid gels to prepare).

In a solid gel matrix, ghost peaks are hard to
eliminate. Also in solid gels, irreversibly-bound
5   material in the matrix tends to interact with the
biomolecules in subsequent separations, thus
decreasing resolution. One advantage of the method
of the present invention is that the separation
matrix, i.e., the polymer/copolymer solution, can be
10   replaced between each run. A further advantage of
the present invention is that when electrokinetic
injection is inefficient in introducing sample mass
into the capillary, a hydrodynamic injection,
accomplished by vacuum or positive pressure, may be
15   used to move a mass of sample into the capillary.

The low-charge-containing polymers and
copolymers used in the method of the present
invention act as coatings of the inner charged
surface wall of the capillary tube and as molecular
20   sieving matrices for biomolecules.

The following examples illustrate, but in no way
are intended to limit the present invention.

25   Example 1
     Synthesis of Charged Polymers or Copolymers
     A.   Copolymer of Acrylamide and TEMED
     The addition of varying amounts of TEMED
(N,N,N',N'-Tetramethyl-ethylenediamine, (Aldrich
30   Chemical Co.) to the ammonium persulfate (Aldrich
Chemical Co.) initiated polymerization of acrylamide
(Aldrich Chemical Co.) results in the production of
polyacrylamides with varying molecular weights (see
viscosity data, Figure 4) and percent charges (see
35   EOF data, Figure 4). The following example

47

illustrates the synthesis of polyacrylamide in which
the molar percent of TEMED to acrylamide is 0.24%.
The molar percent of TEMED can be increased or
decreased by increasing or decreasing the
5   concentration of the TEMED in the solution which is
added to the polymerization mixture in this example.

To 500 mL of deionized water contained in a 2 L
flask is added 100 g of acrylamide. This solution is
stirred for 15 minutes at 50°C with constant but
10   gentle bubbling of helium. Add 400 mL of methanol
(Burdick and Jackson, HPLC grade) and continue
stirring for an additional 15 minutes with a water-
cooled condenser attached to the reaction flask. Add
10 mL of 10% (v/v) TEMED in water and stir for 2
15   minutes. Add 10 ml of 10% (w/v) of ammonium
persulfate and allow polymerization to progress for 2
hours with stirring.

Pour the clear, viscous liquid reaction mixture
into a 3 L polypropylene beaker. With constant
20   manual stirring, slowly add 500 mL of methanol. The
resulting precipitate of solid, white clump(s)of
polyacrylamide are allowed to settle to the bottom of
the beaker. Decant the supernatant to waste. Add
500 mL of methanol to the beaker and manually squeeze
25   the clump(s) with a glass rod and swirl methanol
around the clump(s) in order to wash residual
material into the supernatant which may appear
cloudy. Allow the solid clump to settle and decant
supernatant to waste. Repeat this methanol washing
30   procedure three times or more until the supernatant
appears relatively clear.

Break or cut the solid clump into pieces of
about 1 cm × 2 cm or smaller dimensions and place
these pieces into polypropylene trays. The trays
35   with the separated pieces are put into a vacuum oven

48

(VWR 1430 or equivalent) and dried for 24 hours at
50-60°C at about 30" Hg vacuum (supplied by a Trivac
D2A vacuum pump or equivalent connected to a cooled
vapor trap). The dried pieces of polyacrylamide are
5   ground to a granular powder in a micro-mill (Bel-Art,
VWR Scientific) and stored desiccated in small vials
until used to make a polymer solution.

     B.   Copolymer of Acrylamide and DADMAC
10   The addition of varying amounts of DADMAC
(diallydimethylammonium chloride, Aldrich Chemical
Co.) to the Ammonium persulfate initiated
polymerization of acrylamide results in the
production of polyacrylamides with varying molecular
15   weights (see viscosity data, Figure 5) and percent
charge (see EOF data, Figure 5). The following
example shows how to make a polyacrylamide with a
molar percent of 0.05 DADMAC to acrylamide. The
molar percent of DADMAC may be increased or decreased
20   by increasing or decreasing the concentration of
DADMAC in the solution used in this example.
     The synthesis and recovery of polyacrylamide is
carried out as specified in the above example A.)
except in place of TEMED, once would substitute 10 mL
25   of 1.14 (w/v) DADMAC in water.

     C.   Conversion of Amide Groups in
           Polyacrylamide to Amines
     The conversion of a small percentage of amide
30   groups in polyacrylamide to the corresponding charged
amine group is accomplished using the well-known
Hofmann degradation (Jen). A solution of 40 parts of
5.25% sodium hypochlorite and 2.3 parts of sodium
hydroxide is added over 20 minutes to 355 parts of a
35   20% solution of polyacrylamide in water. The

49

polyacrylamide is prepared as described above in A.) without the addition of TEMED. With a temperature of 30 to 37°C the reaction is held for 30 minutes. The reaction solution is neutralized with HCL to pH 6.9.

5   The polyacrylamide is recovered by precipitation as described above in A.). The percent charge on the polyacrylamide is increased or decreased by increasing or decreasing the total reaction time.

10   D.   Grafting Copolymer of Acrylamide and DADMAC
              onto Dextran
         Acrylamide copolymer is grafted onto a backbone of dextran using a procedure similar to (McCormick). An aqueous solution of 1.25 g dextran, 8.165 g

15   acrylamide, and 0.66 g DADMAC is stirred under helium at 25°C for 30 minutes. Ten mL of 0.05 N nitric acid solution containing 0.0274 g ceric ammonium nitrate is added to start polymerization. After 3 hours the graft copolymer is recovered by precipitation as

20   described in A.). The percent charge is increased or decreased from this example (i.e., 0.05%) by increasing or decreasing the amount of DADMAC added to the reaction mixture.

25   E.   Polymer or Copolymer Solutions
         The polymer or copolymer is added to the desired buffer solution (w/v). The solution is then mixed by gentle rotation for approximately two hours after which the solution is filtered through a 0.45 micron

30   filter. The solution is placed in a vacuum oven for degassing at 30°C and 29 inches of Hg for 15 minutes. The solution is then introduced into the detector-side and buffer-side reservoirs of the capillary electrophoresis instrument.

35

50

Example 2
The Effect of Charged Polymer or Copolymers on Specific Viscosity and Electroosmotic Flow

Capillary electrophoresis was carried out using
5   an ABI Model 270 Capillary Electrophoresis System.
The system includes a built-in high-voltage DC power
supply capable of voltage settings up to 30 KV. The
capillary tube used in the system is a fused silica
capillary tube 50 cm long with a 55 μm i.d. and 350
10   μm o.d.obtained from Polymicro Technologies (Phoenix,
AZ).

The marker used to indicate the rate of
electroosmotic flow (V_{eo}) was the neutral compound,
mesityl oxide, which has a UV absorbance at 200 mm.
15   The electrophoretic system was run at a voltage
setting of about +10 kV (about +200 V/cm) through the
run at a temperature of 30°C. UV detection was with
a built-in 783 UV detector designed for capillary
tube detection. The detector output signal was
20   integrated and plotted on a Spectrophysics Sp4400
integrator/plotter.

Fresh capillary surface was routinely prepared
by flushing the capillary successively with 5-10
capillary volumes of 1.0 N NaOH, 3-5 volumes 0.1 N
25   NaOH, and finally 3-5 volumes of polymer solution.
Solutions are drawn through the capillary by vacuum
using a built-in regulated vacuum system at the
anodic end.

In general, after equilibration with the chosen
30   polymer/copolymer electrolyte solution, 2-5 nl of
neutral marker (mesityl oxide) is injected into the
capillary by virtue of vacuum applied at the detector
end; the marker is used to measure electroosmotic
flow. The field is applied (+200 v/cm) and the time

51

(t, seconds) for the marker to migrate to the detector (30 cm) is recorded.

Electroosmotic flow mobility (EOF) is calculated as:

5

$$EOF = \frac{30 \text{ cm}}{200 \text{ V/cm}} \quad \frac{1}{t}$$

10

Biomolecules are typically electrokinetically injected. For example, a sample of the biomolecules, at about 0.01 milligrams per ml., is electrokinetically injected at -5 kV for 10 secs
15 following electrokinetic injection of the reference marker at -5 kV for 2 seconds. Appropriate voltage is then applied to complete the run, typically of about -10 kV up to approximately -20 kV, depending on the particular separation.

20

A.   Acrylamide and TEMED Copolymer

Mixtures of acrylamide and tetramethylethylene-diamine (TEMED) were formed as described in Example 1 at the TEMED to acrylamide monomer ([AA$_m$]) percent
25 shown at the bottom of Figure 4. The copolymer solution was formed in a buffer consisting of 50 mM ACES, pH 7.0 and 0.2% SDS. This buffer or other suitable electrolyte solutions can be used for formation of the copolymer electrolyte solution. The
30 concentration of polyacrylamide was 2% (w/w). The viscosity of the copolymer electrolyte solution was determined using a capillary viscometer, as described above. The copolymer electrolyte solution was pulled by vacuum through the capillary tube as described
35 above. Two to five nanoliters of neutral marker (mesityl oxide) were injected into the capillary by

52

virtue of vacuum applied at the cathodic end.   A
voltage of 200 v/cm was applied.   Electroosmotic flow
(EOF) was determined as the mobility of the marker
calculated in $cm^2/sec-V$.

5   The results are presented in Figure 4.   As can
be seen from Figure 4, at TEMED/$[AA_m]$ values in the
range of approximately 0.05% to 0.5%, EOF mobility is
reduced to less than 2×10^{-5} $cm^2/sec-V$:   an EOF
mobility value of approximately 2×10^{-5} $cm^2/sec-V$

10   reflects a very low level of electroosmotic flow.

        B.   Acrylamide and DADMAC Copolymer
        Mixtures of acrylamide and diallyldimethyl-
amoniummchloride (DADMAC) were reacted as described

15   in Example 1 at the DADMAC to acrylamide monomer
($[AA_m]$) percent shown at the bottom of Figure 5.
The copolymer solution was formed in a buffer
consisting of 50 mM ACES, pH 7.0 and 0.2% SDS.   This
buffer or other suitable electrolyte solutions can be

20   used for formation of the copolymer electrolyte
solution.   The concentration of acrylamide copolymer
was 2%.   The viscosity of the copolymer electrolyte
solution was determined using a capillary viscometer,
as described above.   The copolymer electrolyte

25   solution was pulled by vacuum through the capillary
tube as described above.   Two to five nanoliters of
neutral marker (mesityl oxide) were injected into the
capillary by virtue of vacuum applied at the cathodic
end.   A voltage of +200 v/cm was applied.

30   Electroosmotic flow (EOF) was determined as the
mobility of the neutral marker calculated in $cm^2/sec-$
V.

        The results are presented in Figure 5.   As can
be seen from Figure 5, at DADMAC/$[AA_m]$ values in the

35   range of approximately 0.05% to 0.26%, EOF mobility

53

is reduced to less than 2 × 10^{-5} cm^2/sec-V: an EOF
mobility value of approximately 2 × 10^{-5} cm^2/sec-V
reflects a very low level of electroosmotic flow.

5   Example 3
                Separation of Sample Polypeptides
            A.   Protein Separation
            A mixture of the following proteins was
purchased from Sigma (St. Louis MO): α-lactalbumin
10   (14 kd), soybean trypsin inhibitor (20 kd), egg
albumin (45 kd), bovine albumin (66 kd), and β-
galactosidase (116 kd). The proteins in the mixture
were SDS-denatured by standard procedures (Ausubel,
et al.). Briefly, the protein at 1.0 mg/ml in 1%
15   SDS/1% mercaptoethanol were heated in boiling water
for 15 minutes. The proteins were diluted with water
to 0.02 mg/ml before analysis.
            Mixtures of acrylamide and diallyldimethyl-
amoniummchloride (DADMAC) were prepared as described
20   in Example 1 at the DADMAC to acrylamide monomer
([AA$_m$]) percent shown in Figures 6 and 7. The
copolymer solution was formed in a buffer consisting
of 50 mM ACES, pH 7.0, (Sigma) which contained 0.2%
sodium dodecyl sulfate (SDS). The concentration of
25   the linear copolymer polyacrylamide/DADMAC in
solution was 2% (w/v). The copolymer electrolyte
solution was pulled by vacuum through the capillary
tube as described above and the cathodic end of the
capillary was placed in the sample mixture. About 1-
30   2 nanograms of protein was injected into the
capillary by virtue of a field strength of -100 V/cm
for 10 seconds. With the capillary end in the buffer
reservoir, a field strength of -200 V/cm was applied.
The separation was run at 30°C and proteins detected

54

at 200 nm. The capillary tube had an inner diameter
of 55 μM and an effective separation length of 28 cm.
      Electropherograms showing the results of CE for
the separation of the components of the protein
5   mixture, performed with DADMAC/[AA$_m$], are shown in
Figures 6 and 7.

      B.   Size Calibration
      The above-described protein mixture was
10 separated essentially as described above, using a
TEMED/[AA$_m$] of 0.24% at a concentration of the linear
copolymer polyacrylamide/TEMED in solution of 3%
(w/v). The log(MW) was plotted versus the migration
of the protein standards relative to α-lactalbumin
15 (migration time of α-lactalbumin divided by the
migration time of the protein peak in question) for
each protein in the mixture and the resulting
calibration curve is shown in Figure 10.

20   C.   Reproducibility
      The reproducibility of the method of the present
invention was demonstrated by repeated sample
injections, under the above conditions, where the
capillary was flushed with the copolymer solution for
25 10 min at 20" Hg between each sample run. All sample
proteins were obtained from Sigma. The concentration
of the linear copolymer polyacrylamide/TEMED in
solution was 3% w/v. The TEMED/[AA$_m$] concentration
was 0.24%. Fifty sequential sample runs were
30 performed and the separation results are shown in
Figure 11 for runs 1, 30 and 50.

      D.   Ferguson Regression Analysis
      The relative migrations of each of the proteins
35 in the above-described protein mixture were

55

determined relative to the migration of α-lactalbumin
at a number of percent copolymer concentrations. The
copolymer used in this analysis was TEMED/[AA$_m$] at
0.24% over the range of concentrations of the linear
5   copolymer polyacrylamide/TEMED in solution shown in
Figure 15. The CE conditions were as described
above.

                          Example 4
10   Separation of Sample Nucleic Acids
          φX174 RF digested with *HaeIII* was purchased from
Bethesda Research Laboratories ((BRL) Gaithersburg
MD). *HaeIII* restriction digestion of φX174 RF yields
11 double-stranded DNA fragments in the size range of
15   72 to 1353 base pairs. The sample was diluted to
0.06 micrograms per microliter. Acrylamide and TEMED
were prepared as described in Example 1 at 0.24 molar
percent TEMED. The copolymer solution was formed in
a buffer consisting of 125 mM TES (Sigma), pH7.0.
20   The concentrations of the linear copolymer
polyacrylamide/TEMED used for nucleic acid separation
are shown in Figure 12 (1, 2 and 3%). The copolymer
electrolyte solution was pulled by vacuum through the
capillary tube as described above and the cathodic
25   end of the capillary was placed in the sample
mixture. About 1-2 nanograms of the DNA restriction
digest mixture was injected into the capillary by
virtue of a field strength of -100 V/cm for 10
seconds. With the capillary end in the buffer
30   reservoir, a field strength of -200 V/cm was applied.
The separation was run at 30°C and the DNA fragments
detected by absorbance at 260 nm. The capillary tube
had an inner diameter of 55 μM and an effective
separation length of 28 cm.

56

An electropherogram showing the results of CE
for the separation of the components of the DNA
fragment mixture, performed with TEMED/$[AA_m]$, are
shown in Figure 12.

5

While the invention has been described with
reference to specific methods and embodiments, it
will be appreciated that various modifications and
changes may be made without departing from the

10   invention.

57

IT IS CLAIMED:

1.   A method of separating biomolecules in a sample comprising

5   preparing a capillary tube with two ends, where the capillary tube (i) has charged chemical groups on its inner wall surface, and (ii) is filled with an electrolyte solution containing 0.05 to 30% weight to weight (w/w) of a non-cross-linked, hydrophilic polymer

10   or copolymer solution containing at least one polymer or copolymer species having (a) a molecular weight between 20 and 5,000 kilodaltons, and (b) a percent charge of between 0.01 to 1.0% as measured by the molar percent of charged monomer subunits to the total polymer subunits,

15   where said charged monomer subunits have the charge opposite to the wall charge at a selected electrophoresis pH,

           immersing the ends of the tube in anodic and cathodic reservoirs containing an electrolyte solution,

20   introducing a sample containing the biomolecules to be separated into one end of the tube, and

           applying an electric field across the reservoirs with a polarity effective to fractionate said

25   biomolecules in the sample.

2.   The method of claim 1, where the polymers or copolymers are selected from the group consisting of polyacrylamides, polyoxides, polyethers, vinyl polymers,

30   cellulose polymers, acrylic polymers, and natural gums and polysaccharides, where said polymers or copolymers have been modified to contain a specified percent charge.

3.   The method of claim 2, where the polymers or

35   copolymers are polyacrylamides selected from the group

58

consisting of polyacrylamide, poly(methacrylamide),
poly(N-methylacrylamide), and poly(N,N-dimethyl-
acrylamide).

5   4.   The method of claim 2, where the polymers or
copolymers are polyoxides selected from the group
consisting of polyethyleneoxide and polypropylene oxide

         5.   The method of claim 2, where the polymers or
10   copolymers are polyethers and the polyether is a
polyvinylmethylether.

         6.   The method of claim 2, where the polymers or
copolymers are vinyl polymers selected from the group
15   consisting of polyvinylpyrollydine, polyvinylalcohol, and
polyvinylacetate.

         7.   The method of claim 2, where the polymers or
copolymers are natural gums or polysaccharides selected
20   from the group consisting of xanthans, dextrans, agar,
guar, and starches.

         8.   The method of claim 2, where the polymers or
copolymers are cellulose polymers selected from the group
25   consisting of methylcellulose, hydroxyethylcellulose,
hydroxylpropylcellulose, and hydroxypropyl-
methylcellulose.

         9.   The method of claim 2, where the polymers or
30   copolymers are acrylic polymers selected from the group
consisting of polyhydroxyethyl-methacrylate and
polyethylene glycol monomethacrylate.

         10.   The method of claim 1, wherein the polymer or
35   copolymer solution contains a homopolymer.

59

11.   The method of claim 1, wherein the polymer or
copolymer molecules contain at least one charged group
selected from the group consisting of primary amines,
secondary amines, quaternary amines, carboxylic acids,
sulfonic acids, phosphoric acids, sulfuric acids, and
phosphonic acids.

12.   The method of claim 11, where the polymer or
copolymer molecules contain a positively charged group
derived from a compound selected from the group
consisting of: diallydimethylammonium salt, tetramethyl-
ethylenediamide, [3-(methacryloamino)propyl]-trimethyl-
ammonium salt, 2,2'-azobis(2-methylpropionamidine) salt,
and 2-methylacryloxyethyl(trimethylammonium) salt.

13.   The method of claim 1, wherein the polymer is a
copolymer of acrylamide and diallyldimethylammonium-
chloride (DADMAC) and said copolymer has a molecular
weight between about 200 and 600 kd, and the polymer
molecules contain 0.02 to 0.4% of the quaternary amine
N,N-dimethyl-pyrrolidine per subunit acrylamide.

14.   The method of claim 1, wherein the polymer is a
copolymer of acrylamide and tetramethylethylenediamine
(TEMED), and said copolymer has a molecular weight
between about 100 and 500 kilodaltons, and the polymer
molecules contain 0.05 and 0.5% of the tertiary amine
tetramethylethylenediamine per subunit acrylamide.

15.   The method of claim 1, wherein the polymer is a
copolymer of N,N-dimethylacrylamide and
tetramethylethylenediamine (TEMED) and said copolymer has
a molecular weight between about 200 and 800 kilodaltons,
and the polymer molecules contain 0.03 to 0.6% of the

60

tertiary amine tetramethylethylenediamine per subunit
N,N-dimethylacrylamide.

16. The method of claim 1, wherein the polymer is a
copolymer of acrylamide and [3-(methacryloylamino)-
propyl]trimethylammonium chloride and said copolymer has
a molecular weight between about 200 and 600 kilodaltons,
and the polymer molecules contain 0.01 to 0.2% of the
quaternary amine N-[(propyl)trimethylammonium chloride]-
methacrylamide per subunit of acrylamide.

17. The method of claim 1, wherein the
concentration of polymer molecules containing a charge
opposite the wall charge is sufficient to non-covalently
coat the wall surface and significantly control and
reduce electroosmotic flow to less than about 2×10^{-5}
cm^2/sec-V.

18. The method of claim 1, which further includes
detecting the presence of separated biomolecules in the
electrophoresis capillary tube by measuring
electrochemical, optical or radioisotopic properties of
the biomolecules in the tube.

19. The method of claim 1, wherein said
biomolecules are selected from the group consisting of
proteins, polypeptides, and peptides.

20. The method of claim 19, where said biomolecules
have been denatured with sodium dodecylsulfate and sodium
dodecylsulfate is present in the electrolyte solution.

21. The method of claim 1, wherein said
biomolecules are nucleic acid fragments.

61

22.   The method of claim 21, wherein the nucleic
acid fragments are denatured with urea or formamide and
the urea or formamide is present in the electrolyte
solution.

5

23.   The method of claim 21, wherein the fragments
are double-stranded nucleic acids, and said differential
migration is adjusted by the addition of an intercalating
agent to the fragments, to increase preferentially the
10   migration rates of smaller molecular weight fragments
through the polymer solution.

24.   The method of claim 21, for use in performing
restriction digest analysis of a DNA sample, which
15   further comprises digesting the sample with one or more
selected restriction endonucleases.

25.   The method of claim 21, where the nucleic acid
fragments are the products of DNA sequencing reactions,
20   and where the DNA sequencing reactions contain nucleic
acids labelled with fluorescent tags.

26.   The method of claim 21, where the biomolecules
are single-strand DNA and the relative migration of the
25   biomolecules is dependent on conformational polymorphisms
between the biomolecules.

27.   The method of claim 21, where said biomolecules
are DNA molecules which are the amplification products of
30   polymerase chain reactions.

28.   The method of claim 1, where said biomolecules
are nucleic acid-protein complexes.

62

29.   The method of claim 1, wherein said bio-
molecules are selected from the group consisting of
linear, branched, native and chemically modified
oligosaccharides.

5

30.   A filled capillary tube for use in capillary
electrophoresis, comprising a capillary tube having
charged chemical groups on its inner wall surface, and
filled with an electrolyte solution containing 0.05 to
10   30% weight to weight (w/w) of a non-cross-linked,
hydrophilic polymer or copolymer solution containing at
least one polymer or copolymer species having (a) a
molecular weight between 20 and 5,000 kilodaltons, and
(b) a percent charge of between 0.01 to 1.0% as measured
15   by the molar percent of charged monomer subunits to the
total polymer subunits, where said charged monomer
subunits have the charge opposite to the wall charge at a
selected electrophoresis pH.

20   31.   The tube of claim 30, wherein the polymers or
copolymers are selected from the group consisting of
polyacrylamides, polyoxides, polyethers, vinyl polymers,
cellulose polymers, acrylic polymers, and natural gums
and polysaccharides, where said polymers or copolymers
25   have been modified to contain a specified percent charge.

32.   The tube of claim 30, wherein the polymer or
copolymer molecules contain a charged group which has a
charged group selected from the group consisting of
30   primary amines, secondary amines, quaternary amines,
carboxylic acids, sulfonic acids, phosphoric acids,
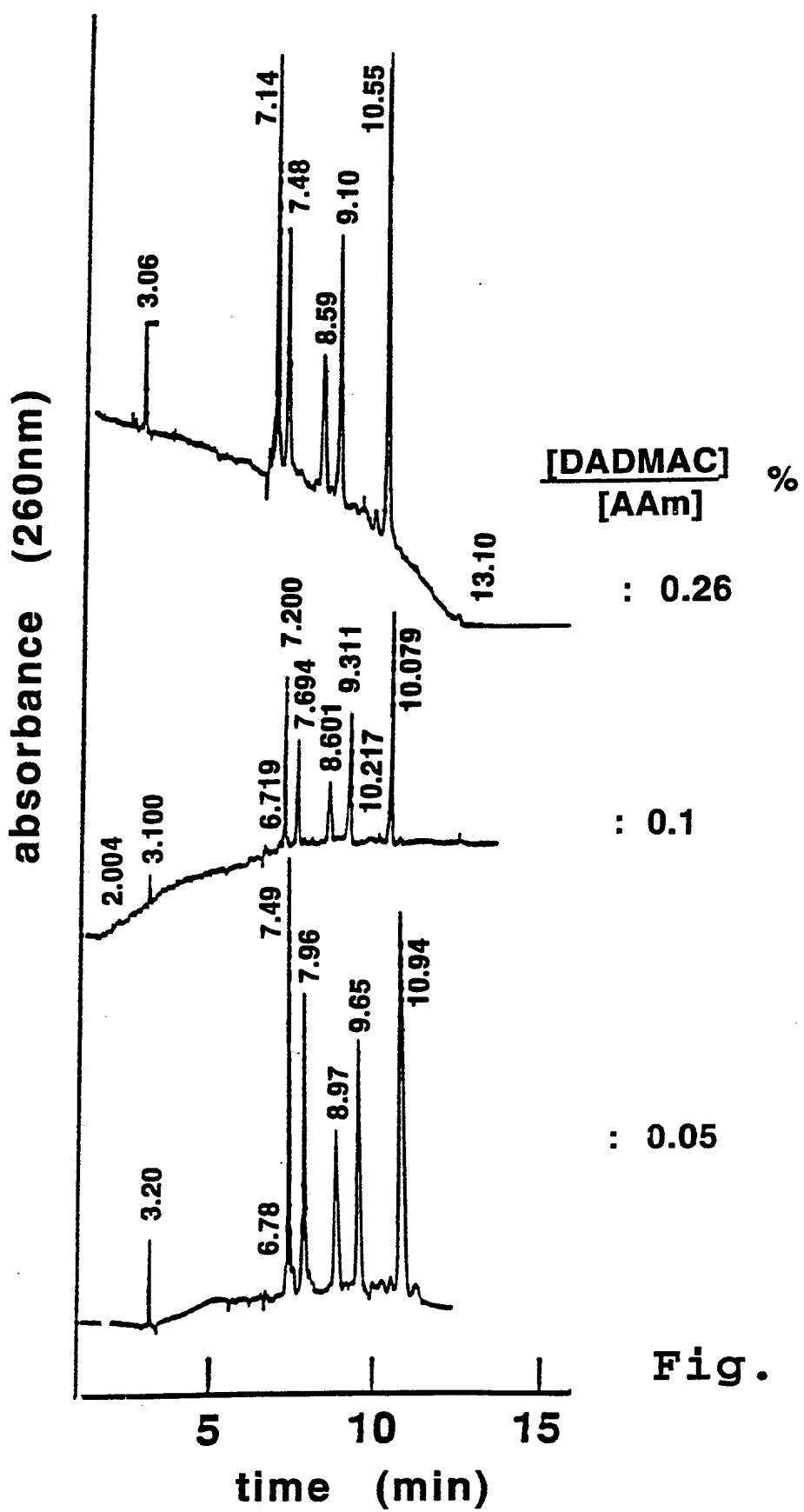sulfuric acids, and phosphonic acids.

1/11
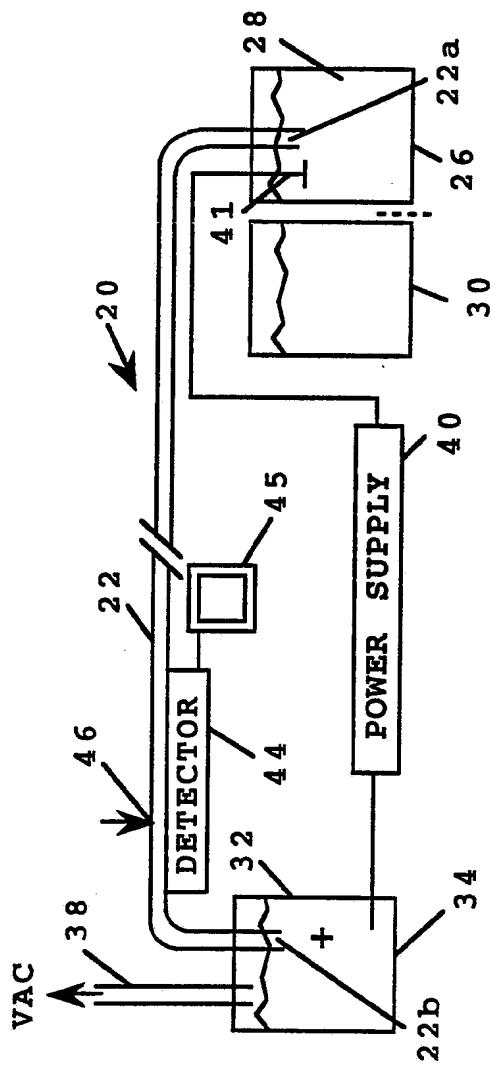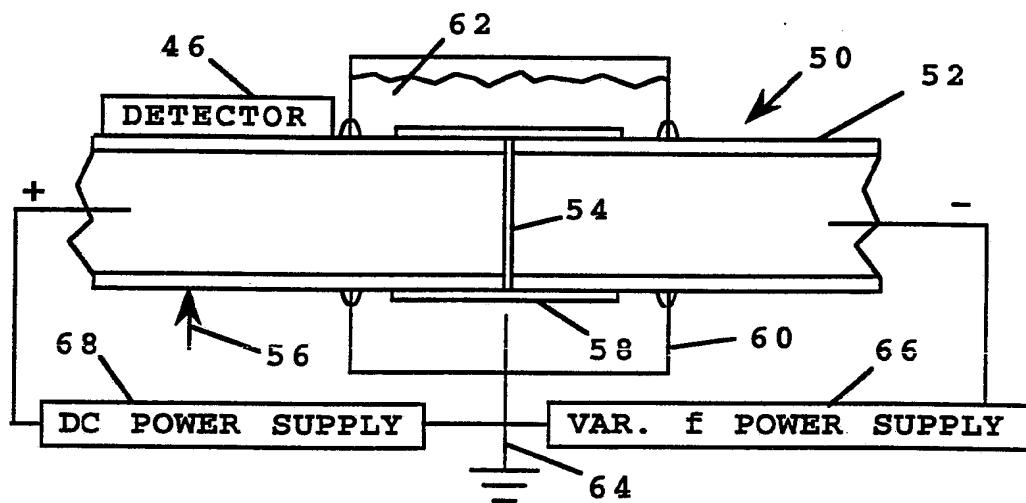
Fig. 1

Fig. 2

Fig. 3

3/11

Fig. 4
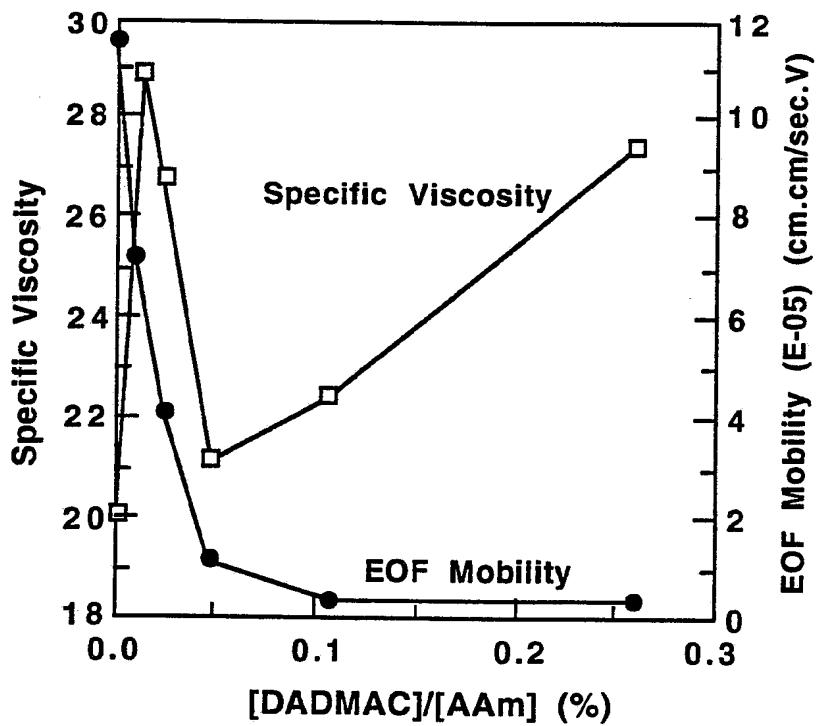
Fig. 5
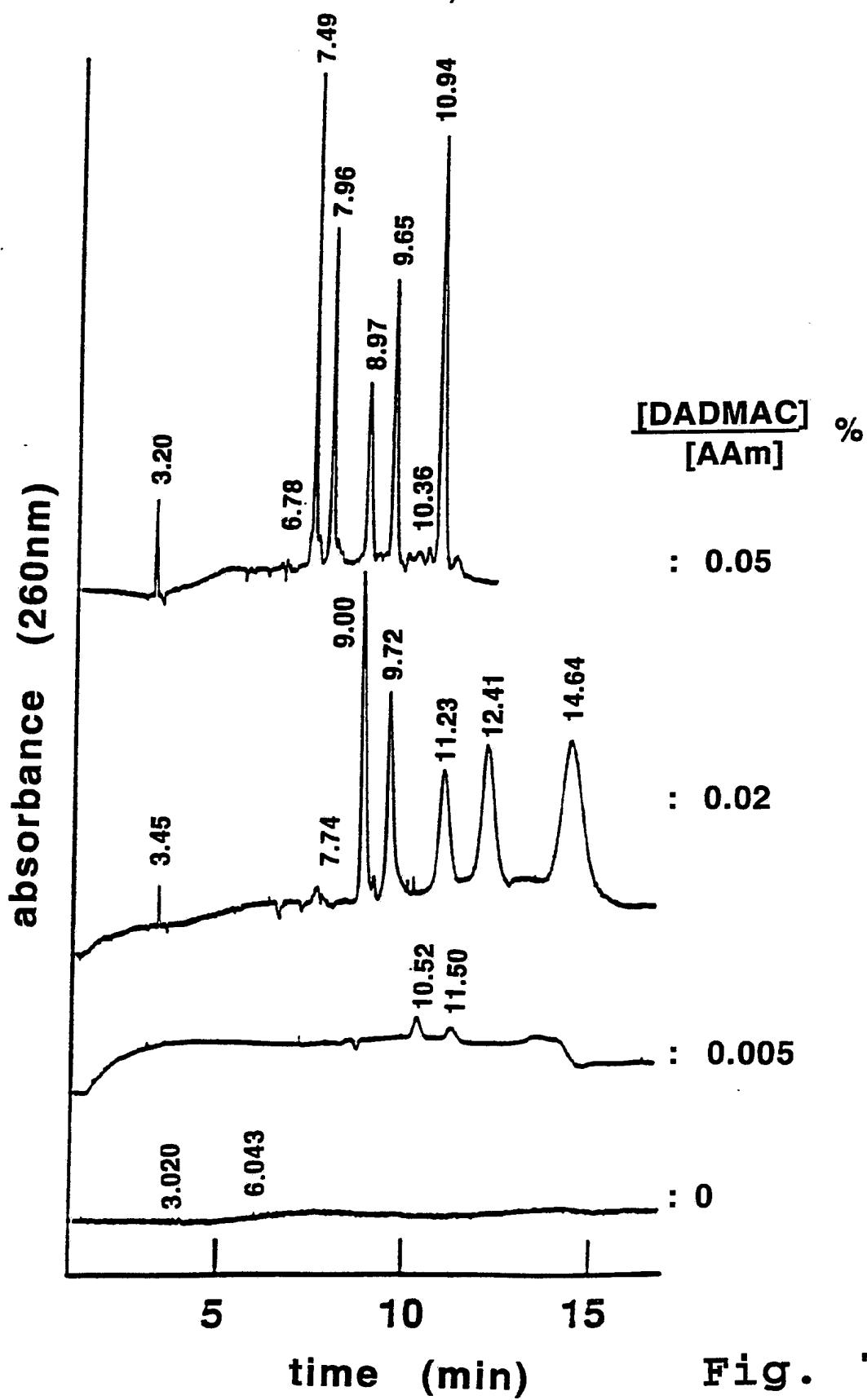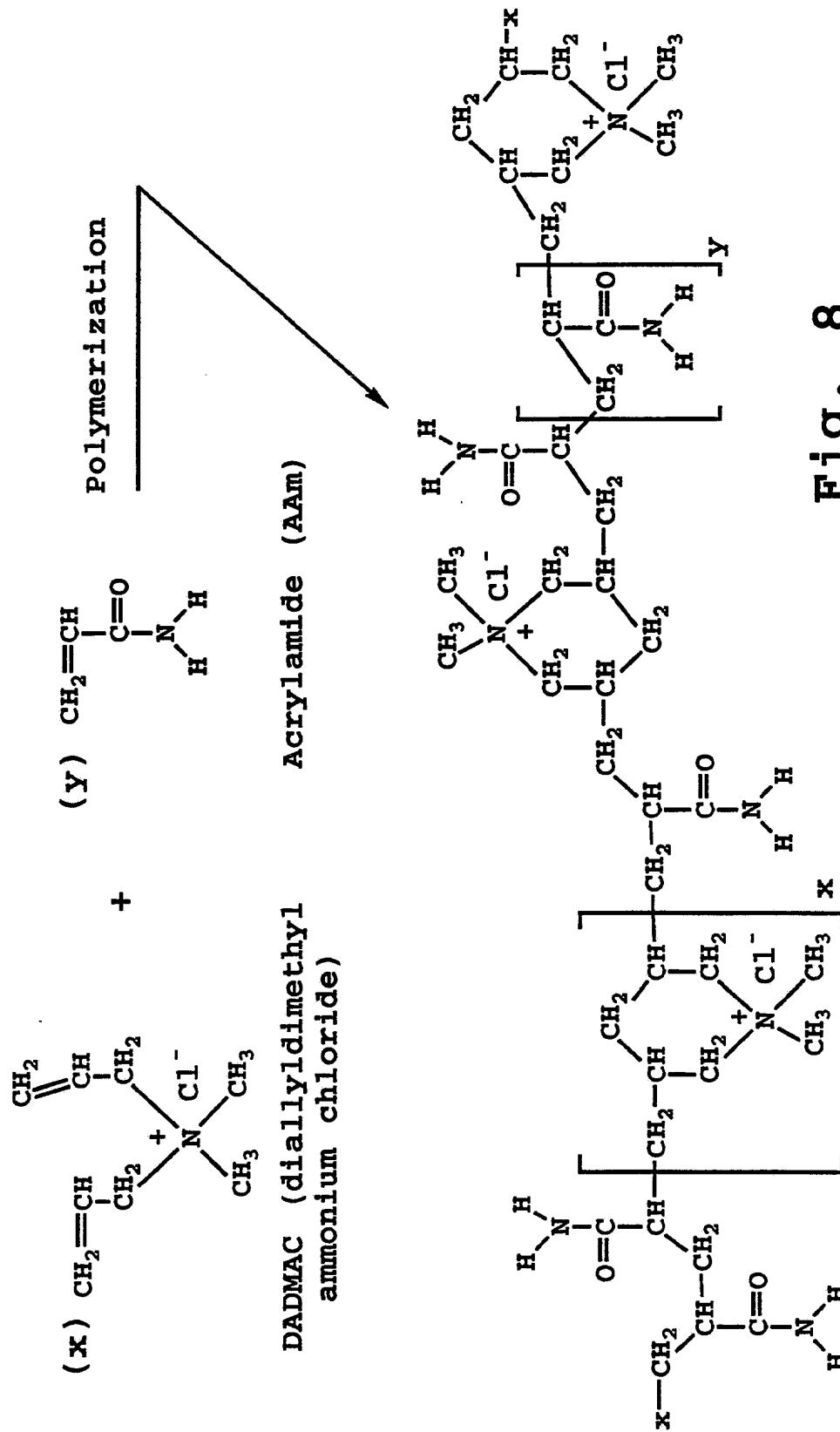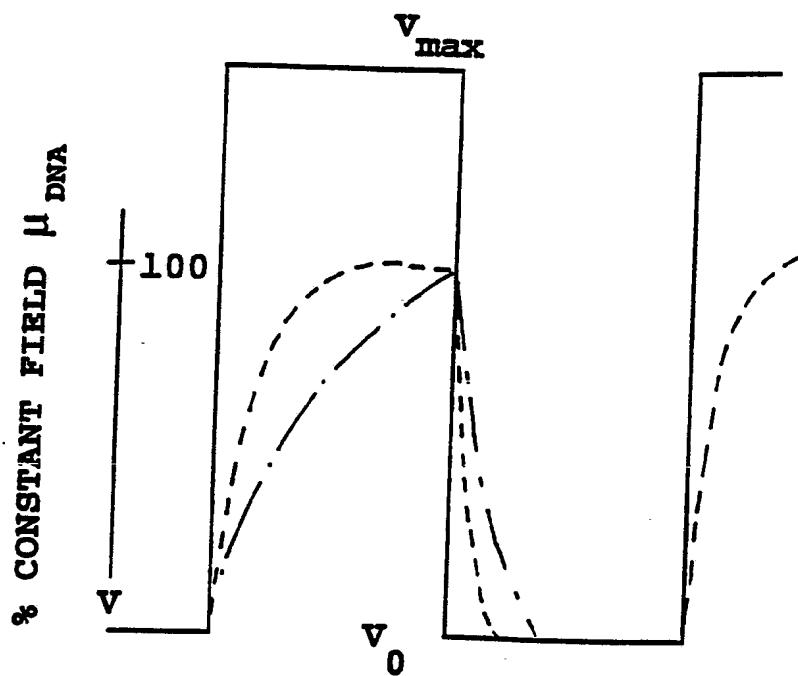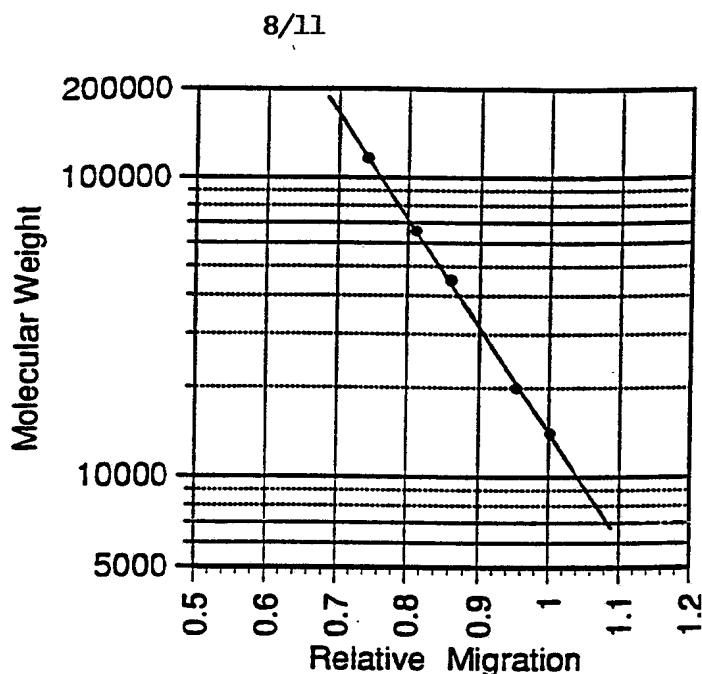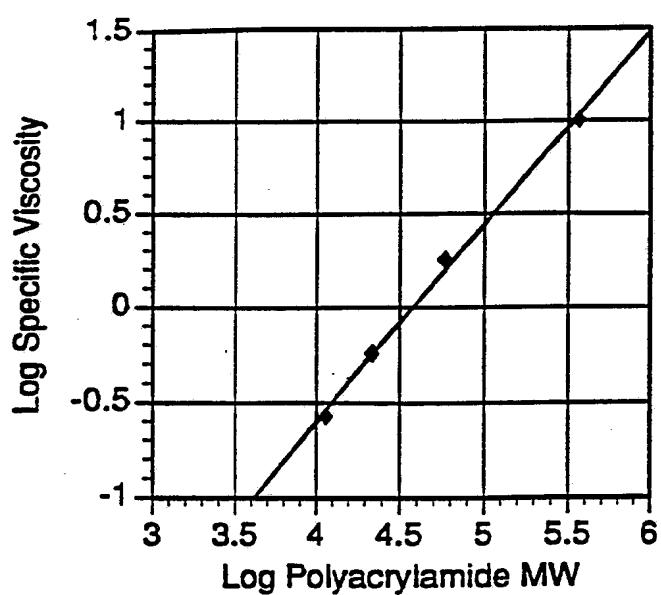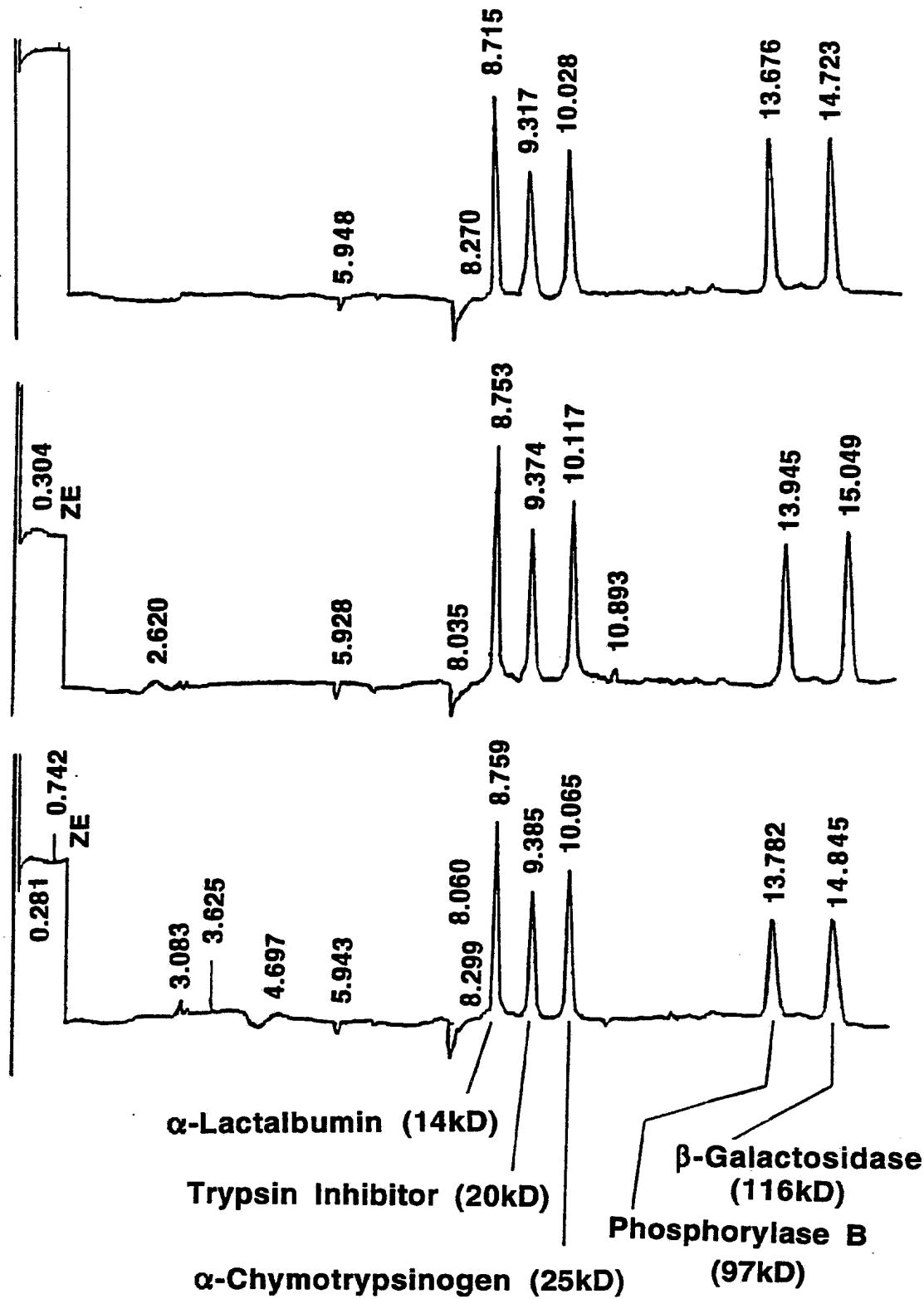
Fig. 6

Fig. 7

Fig. 8

Fig. 9

Fig. 10

Fig. 13

Fig. 11

Fig. 12

Fig. 14

□ Trypsin Inhibitor
○ Ovalbumin
▲ Bovine Albumin
● β-Galactosidase
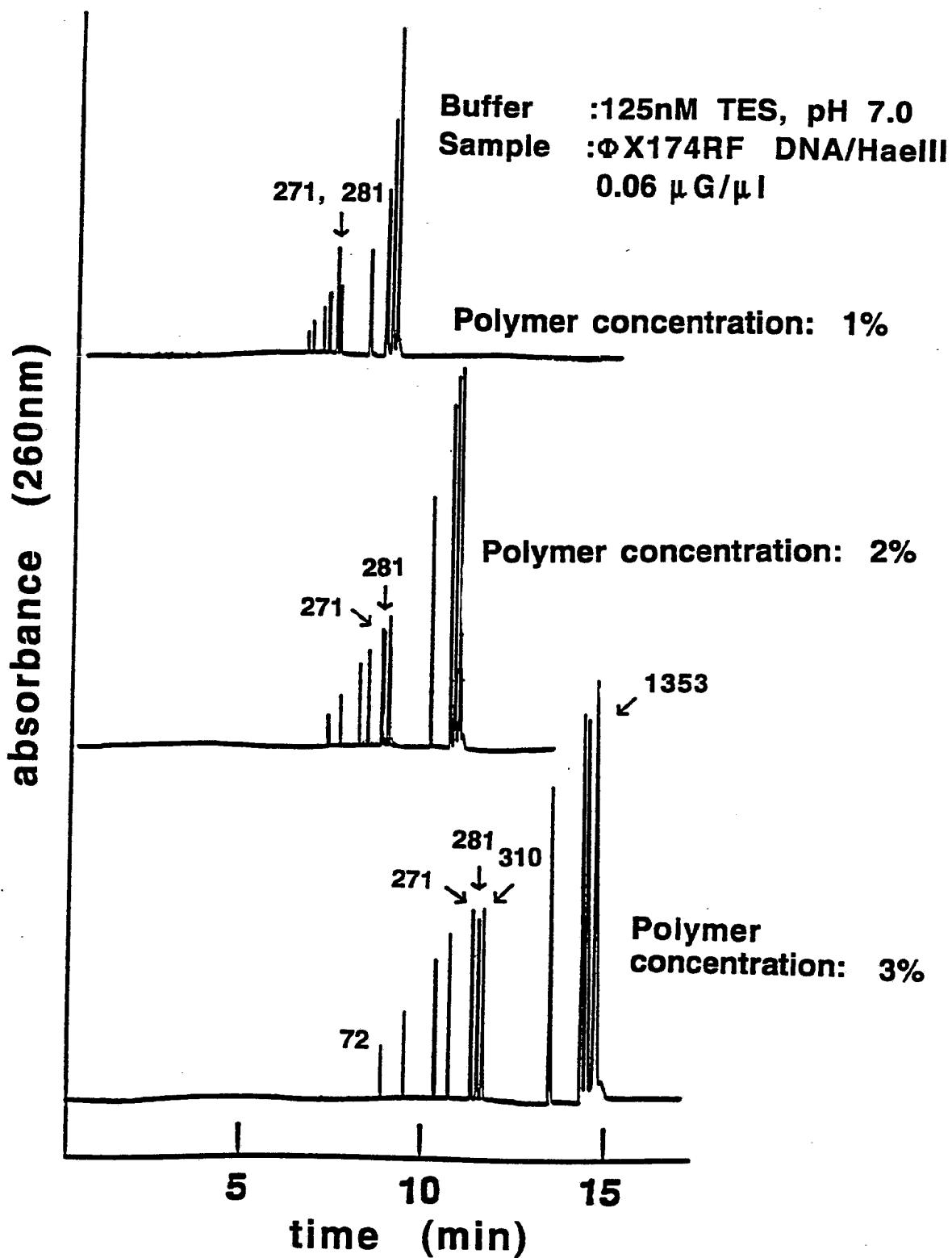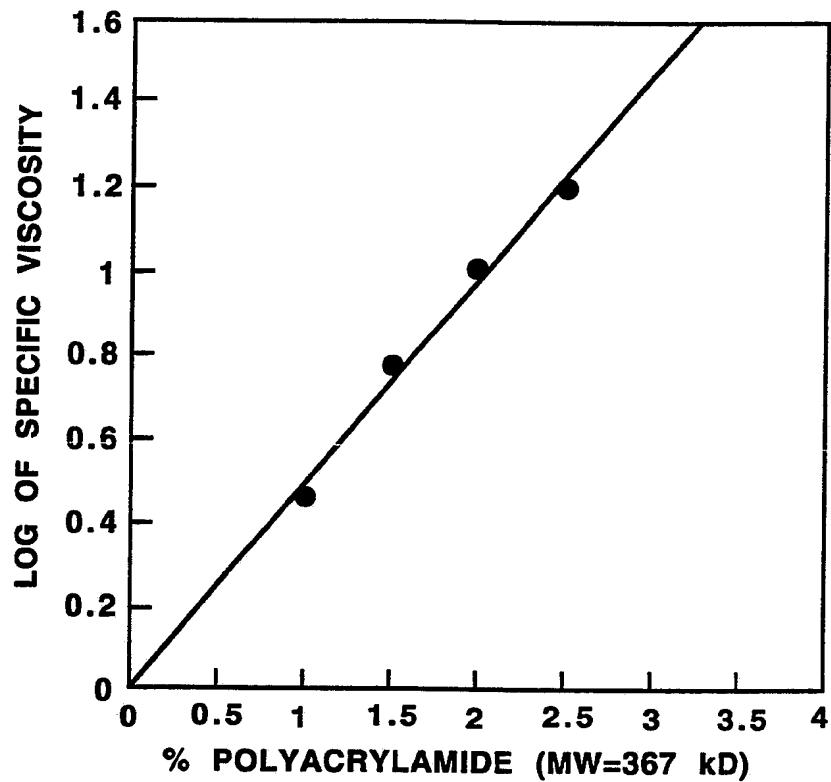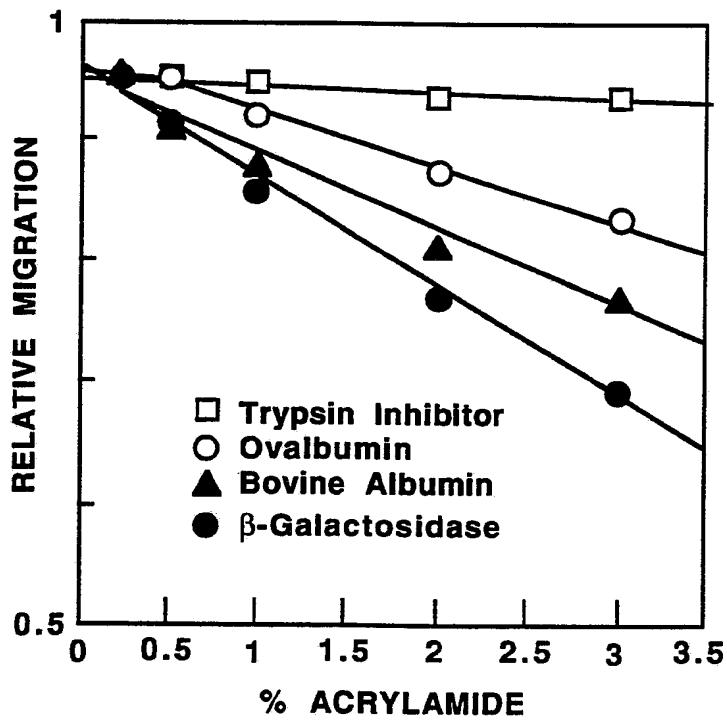
Fig. 15

INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :G01N 27/26, 27/447

US CL :204/180.1, 299R, 182.8

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. :

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Journal of Chromatography, Volume 549, (1991), Alexandra widhalm et al, "Capillary zone electrophoresis with a linear, non-cross-linked polyacrylamide gel: separation of proteins according to molecular mass", 446-451, see "procedure" section page 447.	1,11-13,15,16,17-22,25-27,29and31
X	Electrophoresis 1991, 12 (1991), Toshio Takagi et al, "Application of schlieren optics to real-time monitoring of protein electrophoresis in crosslinker-free linear polyarcylamide solution", 436-438, see page 437.	1,11-13,15,16,17-22,25-27,29 and 31

☐ Further documents are listed in the continuation of Box C.   ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be part of particular relevance		
"E"	earlier document published on or after the international filing date	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)		
"O"	document referring to an oral disclosure, use, exhibition or other means	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"P"	document published prior to the international filing date but later than the priority date claimed	"&"	document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
28 JUNE 1993	27 JUL 1993

Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	Authorized officer JOHN S. STARSIAK
Facsimile No. NOT APPLICABLE	Telephone No. (703) 308-0661

Form PCT/ISA/210 (second sheet)(July 1992)*